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Pathogen Induced Hypersensitive Response and Systemic Acquired Resistance in the Moss Amblystegium serpens

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Pathogen Induced Hypersensitive Response and Systemic Acquired Resistance in the Moss *Amblystegium serpens*

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DEDICATION

This thesis is dedicated to Dr. Philip Villani. He is an excellent mentor, teacher, and friend that opened my eyes to the wonders of the plant world. I cannot thank you enough.

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ABSTRACT

The moss *Amblystegium serpens* and the pathogenic fungus *Pythium irregulare* were used to study plant-pathogen interactions in a non-vascular plant. The major findings in this report include that *P. irregulare* does infect *A. serpens* but entire moss death was not noted, 25°C is the optimum temperature for *P. irregulare* appressoria formation, most appressoria were found on *A. serpens* leaves, and the SAR response within *A. serpens* seems to be reducing the number of *P. irregulare* penetrated moss cells. Future research on the pathogen defense mechanisms of *A. serpens* is important and promising. When we elucidate these complex chemical processes in "simpler" model systems such as *A. serpens*, we can then apply this information to increase the yields of economically important agricultural plants such as corn (*Zea mays*) and soybean (*Glycine max*).

ABBREVIATIONS

ECC 7: *Erwinia carotovora* ssp. *carotovora* strain 7

ECC 71: *Erwinia carotovora* ssp. *carotovora* strain 71

HR: Hypersensitive Response

SAR: Systemic Acquired Resistance

JA: Jasmonic Acid

SA: Salicylic Acid

LB: Luria Bertani medium

PDA: Potato Dextrose Agar

TRIS: Tris(hydroxymethyl)aminomethane

cAMP: Adenosine 3', 5' Monophosphate

GC-MS: Gas Chromatography-Mass Spectrometry

BSA: Bovine Serum Albumin

A. serpens: *Amblystegium serpens*

P. irregulare: *Pythium irregulare*

C. purpureus: *Ceratodon purpureus*

P. patens: *Physcomitrella patens*

P. aeruginosa: *Pseudomonas aeruginosa*

A. sympodiale: *Acarosporium sympodiale*

P. vexans: *Pythium vexans*

A. muscivora: *Atradidmyella muscivora*

A. thaliana: *Arabidopsis thaliana*

F. hygrometrica: *Funaria hygrometrica*

INTRODUCTION

In order to adequately study host-pathogen interactions, a host and pathogenic organism must be selected. Then, the lifecycles of both of these organisms must be learned about. Finally, information concerning plant defenses against pathogens must be uncovered. These fundamental steps allowed this research to be undertaken.

The Microbial World

Invisible to the naked eye is a microbial world of immense diversity. Both animals and plants are surrounded and sometimes colonized by these microscopic organisms. On and in the human body alone, it is thought that about 10^{11} microorganisms thrive, and for every one human cell there are ten bacterial cells (Rosebury, 1969; Nester *et al.*, 2009). Moreover, about 10,000 different species of Archaea and bacteria can be present in one gram of soil; this figure does not take into account the fungi present (Staley, 2007). Many of these microorganisms are beneficial and support the lives of many other organisms. For example, normal human intestinal flora allow for food to be digested and aid in preventing harmful bacteria from colonizing the intestines (Nester *et al.*, 2009). Unfortunately, we almost always overlook these beneficial organisms.

A small number of pathogenic microorganisms are well known. Some are host specific to animals, whereas, some colonize plants. However, it is rare that a pathogen can infect both animals and plants. Specifically, a pathogen is any bacterium, fungus, or virus capable of causing disease with potentially unpleasant symptoms. This ability to cause disease stems from numerous microbial aspects, or virulence mechanisms, that allow the microbe to evade or penetrate the organism's defense mechanisms. For

example, a thick, hydrophobic mycollic acid layer surrounding *Mycobacterium tuberculosis* hinders human immune cell phagocytosis. Moreover, pathogenic fungi produce hydrolytic enzymes that break down cellulose of plants. This allows for the cell wall to be punctured and nutrients to be obtained from the cytoplasmic contents. Finally, the bacterium *Erwinia carotovora* has enzymes that break down pectin and cause soft-rot in many plant species, including potato (*Solanum tuberosum*) (Ponce de León *et al*., 2007). Clearly, the number of pathogenic microorganisms is small, but they have ingenious virulence mechanisms to invade and circumvent host defense mechanisms.

Human Defense Against Pathogens

In humans, both innate and acquired responses protect against these pathogens. Innate mechanisms protect indiscriminately against any microbe. The skin, mucous membranes, cough reflex, hydrolytic enzymes, and low pH of the stomach are some innate defenses. However, if a microbe thwarts these physical barriers and chemical defenses, acquired defense mechanisms take over. Only found in vertebrates, the acquired response involves immune system cells specifically recognizing and producing antibodies against the foreign microbe. Simply, the immune system is a collection a cells that flow through the circulatory systems waiting to encounter foreign microbes. Clonal selection occurs when antigens on the microbe interact with antibodies on an immune system B-cell, which then proliferates rapidly. Also, T-cells are sometimes recruited to aid in the response. Eventually, an abundance of antibodies are produced that help aggregate the microbe and allow for phagocytosis. Memory cells are also formed to prevent future infections from the same microbe. Overall, human defense against

pathogenic microbes first consists of physical and chemical barriers, and then complex cellular interactions are employed (Coico and Sunshine, 2009).

Non-vascular Plant Pathogen Defense

Vascular and non-vascular plants are two groupings of plants based on the presence or absence of transport tissues. Specifically, vascular plants possess tissues that transport water and nutrients called xylem and phloem; these allow plants to grow large. Some examples include economically important plants such as food crops and trees. On the other hand, non-vascular plants do not possess these transport tissues and do not grow as large. Some examples are mosses, lichens, liverworts, and algae.

It is important to note the similarities and differences between non-vascular plant and human pathogen defense. First, non-vascular plant pathogen defense is of primary importance because plants are not able to move and evade pathogens. However, humans can evade pathogens. Also, similarities between human and non-vascular plant innate defenses are present. For example, non-vascular plants have physical barriers including the epidermis that parallels the function of human skin. Of note is that cell wall reinforcement through cellulose production and cuticle thickening occurs after pathogenic exposure. Moreover, both humans and non-vascular plants have antimicrobial compounds that help defend against invaders. Plants use a class of antimicrobial compounds called phytoalexins. However, non-vascular plants do not have an acquired immunity system like humans. Instead of the immune cells used by humans, non-vascular plants use resistance (R) proteins that trigger two chemical processes to combat pathogens that evade the innate defenses (Campbell and Reece, 2005; He, 1996; Oliver *et al*., 2009).

Of fundamental importance is the recognition of the pathogen by the plant. R proteins, which are receptor proteins produced by the plant, have specificity to certain pathogen antigens. Simply, an antigen is a molecular shape on the outside of the pathogen. If an R protein binds to a pathogenic antigen, signal transduction within the cell occurs and triggers the enhancement of the chemical processes called hypersensitive response (HR) and systemic acquired resistance (SAR). Unfortunately, the exact molecular mechanism by which R proteins trigger HR and SAR is not fully understood. But, this is an aim of current research (Campbell and Reece, 2005).

HR and SAR are the two main plant pathogen defense mechanisms. HR is a localized response that prevents the pathogen from spreading cell to cell by intentionally killing healthy cells around the site of infection and increases cell wall thickness. To accomplish this, the plant produces reactive oxygen species, phytoalexins, and cellulose to increase cell wall thickness. Once the pathogen is limited to the site of infection, adjacent plant cells undergo apoptosis, or programmed cell death. Fundamentally, pathogens require living plant cells in order to access water, nutrients, and propagate throughout the organism. Without the presence of living cells, the pathogen is effectively quarantined, while ensuring the survival of the rest of the organism (Ponce de León *et al*., 2007). The localized HR response is effective, but a broader response is needed. HR triggers SAR, which prevents future pathogen infections in a nonspecific manner. During HR, plant chemical signals are released that function to initiate SAR. These proposed chemicals include jasmonic acid (JA), salicylic acid (SA), and/or ethylene (Oliver *et al*., 2009). Unfortunately, the cellular mechanisms that prevent future infection are not fully

understood. However, it has been noted that the SAR response lasts for days. (He, 1996; Campbell and Reece, 2005).

Amblystegium serpens Lifecycle and Morphology

Amblystegium serpens was the moss chosen to research. It is in the division Bryophyta and the order Bryospida. It is important to understand the moss lifecycle (Fig. 1) and the structural morphologies of this organism. Interestingly, mosses have both haploid and diploid stages in their lifecycle. Haploid means having only one set of chromosomes; whereas, diploid means having a set of paired chromosomes. First, haploid spores are released from a structure called a sporangium. These spores germinate to form filamentous protonemata. At a certain point, a bud forms from a protonema and grows into a gametophyte; there are male and female gametophytes. This is the moss lifecycle stage that you notice outside. Male gametophytes have reproductive structures

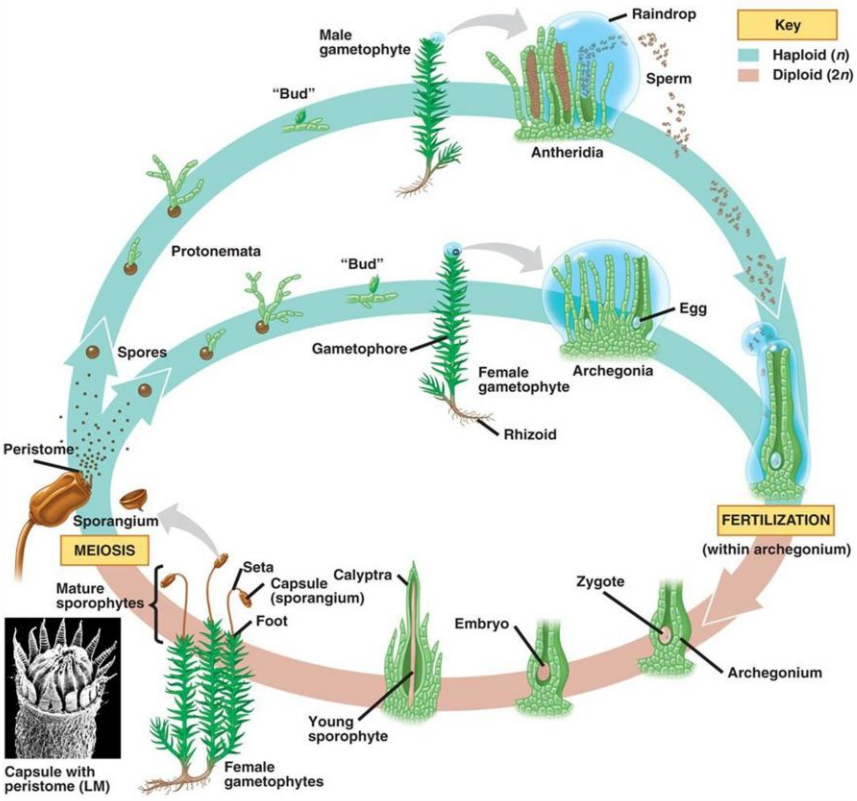

Figure 1. A representative moss lifecycle (Campbell and Reece, 2005).

called antheridia, whereas female gametophytes have archegonia. Water is necessary for sperm from the antheridium to swim to the eggs in the archegonium. Fertilization then occurs. This marks the beginning of the diploid stage. The embryo grows and forms into a sporangium, which undergoes meiosis on the inside to produce spores. The process then repeats (Schofield, 1985). Having both significant haploid and diploid life stages is unique. For this research, *A. serpens* gametophytes were used.

Figure 2. *Amblystegium serpens* culture. Note morphological characteristics such as spreading growth pattern, multiple stems, small leaves, and brown rhizoids.

A. serpens gametophytes are small but have unique morphologies. *A. serpens* is a spreading moss that has green stems branching over the surface of the growing medium (Fig. 2). Small, green leaves with pointed tips radiate out from the stems. Moreover, brown, filamentous structures called rhizoids intermittently emerge from the stem. Rhizoids function similarly to roots in that they help support the organism and are

involved in nutrient absorption. Overall, the stems, leaves, and rhizoids are the major macroscopic structures of *A. serpens*.

To the best of my knowledge, only a handful of studies have investigated the infection of non-vascular plants by pathogens. For example, *Pythium irregulare, Pythium debaryanum* (fungi), and *Erwinia carotovora* ssp. *carotovora* (a bacterium) infect *Physcomitrella patens* (Oliver *et al*., 2009; Ponce de León *et al*., 2007). *Atradidymella muscivora* (a fungus) infects *Funaria hygrometrica, Polytrichum juniperinum,* and *Hylocomium splendens* (Davey *et al*., 2009). Furthermore, non-vascular plant pathogen defense research of HR and SAR is very limited (Minibayeva and Beckett, 2001; Ponce de León *et al*., 2007). Of the literature examined, only one moss (*P. patens*) has undergone extensive research (Oliver *et al*., 2009; Ponce de León *et al*., 2007). Why is this the case? Fundamentally, non-vascular plants are not as economically important as vascular plants; thus, they are the focus of less research. Overall, learning about preventing pathogen infection to ensure non-vascular plant survival is not a big concern for scientists at the present time. This is unfortunate, because additional research is warranted. The question I wonder about is whether or not other species behave similar to *P. patens* in their response to pathogens?

An ideal host model for pathogen defense studies is *A. serpens*. I chose *A. serpens* (a ubiquitous moss) because it grows quickly and can be propagated easily on artificial medium. Additionally, no research has been done on pathogen infection or defense responses in this species, and *A. serpens* is in a different taxonomic group than *P. patens* (the spreading mosses verses the upright mosses, respectively).

Pythium irregulare Lifecycle and Morphology

Pythium irregulare is a white, filamentous, pathogenic fungus in the division Mastigomycota and the class Oomycetes that has a worldwide distribution (Middleton, 1943). This fungus is an ideal plant pathogen to use. First, it has a large host range (more than 200 plants) and grows quickly on an artificial media, including potato dextrose agar (PDA). Second, the one report I found that investigated non-vascular plant pathogen defense utilized *P. irregulare* (Oliver *et al*., 2009).

To better understand how this fungus infects plants, a greater understanding of its lifecycle is needed (Fig. 3). Interestingly, fungi have both sexual and nonsexual lifecycles. First, filamentous structures called mycelia produce zoosporangia that mature into zoospores. The zoosporangium is analogous to sporangium in mosses. Spores are then released and germinate to produce mycelia. Second, a sexual lifecycle can be entered. From the mycelia, male reproductive structures called multinucleated antheridia, and female reproductive structures called oogonia can form. Then, meiosis occurs to form uninucleated antheridia, which undergo further maturation and cellular differentiation. In the end, mature oospores are formed and germinate to produce mycelia. Overall, it can be seen how the mycelium is the structure that both the nonsexual and sexual lifecycles stem from (Moore-Landecker, 1990).

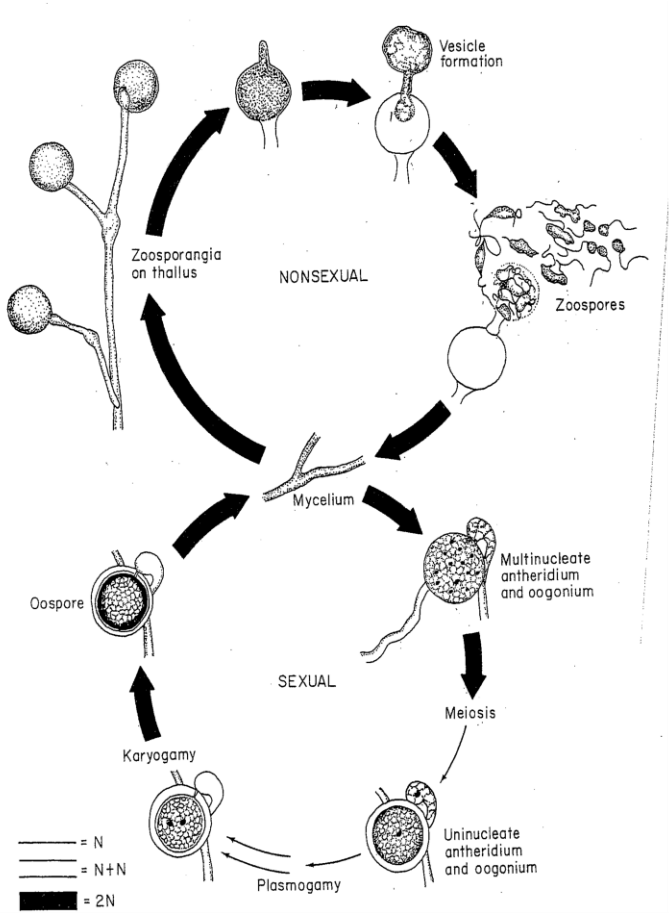

Figure 3. *Pythium debaryanum* fungus lifecycle (Moore-Landecker, 1990).

Appressoria: The Infection Structures

During host infection, structures called appressoria can form (Fig. 10). The morphologies of these structures vary. *P. irregulare* has bulbous, circular appressoria at the tips of the mycelia. Where an appressorium makes contact with the plant cell wall, hydrolytic enzymes called cellulases are produced that break apart cellulose molecules in the plant cell wall, weakening the cell wall (Davey *et al*., 2009). Then, hydrostatic pressure within the appressorium increases, while an infection peg is produced. The hydrostatic pressure increases until the infection peg is forced through the weakened cell wall, and the fungal hypha gains access to the cell.

Thesis Hypothesis

I hypothesize that *P. irregulare* will infect *A. serpens*. This will cause necrosis, chloroplast degradation, and stem browning in *A. serpens*. Yet, *A. serpens* will exhibit pathogen defense mechanisms (HR and SAR) that limit destruction caused by the pathogen. Research on this specific moss and fungus interaction is significant because it will add to the limited knowledge of pathogen interactions and responses in non-vascular plants.

MATERIALS AND METHODS

Obtaining and Growing Amblystegium serpens, Erwinia carotovora ssp. carotovora, Pseudomonas aeruginosa, and Fungi

A. serpens capsules, obtained from the Butler University, Indianapolis, IN, campus by Meghan Knight in the summer of 2008, were sterilized, germinated, and aseptically grown on BCD medium (Fig. 4). 1.0L of BCD medium was prepared by mixing 10mL of each stock solution B, C, D, 8.0g of agar, and distilled H_2O (Table 1). The medium was brought to a boil in a microwave, autoclaved, and poured into petri dishes. *A. serpens* was grown at 25°C under a fluorescent lightning system that provided 16 hours of light a day (Fig. 5). Experimentation was carried out on 2 or 3 week old cultures. Using flame sterilized instruments and a laminar airflow hood, subculturing 2cm long pieces of *A. serpens* was carried out as needed.

Figure 4. *A. serpens* growing aseptically on a petri dish.

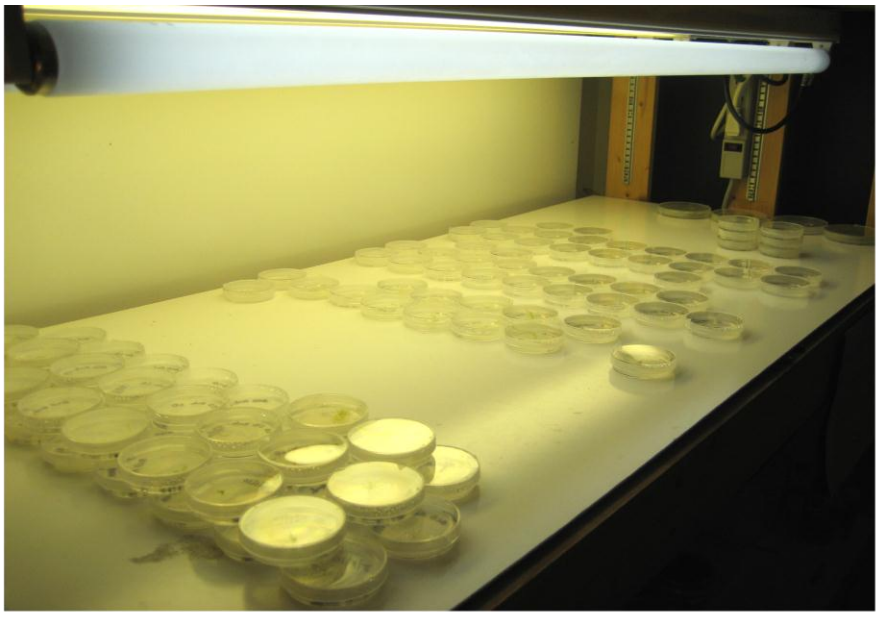

Figure 5. *A. serpens* growing under a fluorescent lightning system that provided 16 hours of light a day.

Erwinia carotovora ssp. *carotovora* strains 7 and 71 (ECC 7 and 71) were obtained from the Division of Plant Sciences at the University of Missouri and cultured on Luria Bertani (LB) broth medium (Table 1). Liquid LB medium was made by mixing 10.0g of Tryptone, 5.0g of Yeast Extract, 10.0g of NaCl, and distilled H_2O to 1.0L. This was then brought to a boil in a microwave and autoclaved. Solid LB medium contained 7.5g of agar added to the liquid LB ingredients. This was brought to a boil in the microwave and then autoclaved. Both media were poured into test tubes. Bacterial solutions were used for experimentation after 12 hours of incubation at 28°C.

P. aeruginosa was obtained from the microbiology lab at Butler University and cultured in liquid and on solid LB media (Table 1). Bacterial solutions were used for experimentation after 12 hours of incubation at 37°C.

The fungi *A. sympodiale*, *A. muscivora*, and *Kretzschmaria* spp. were obtained from the University of Alberta Microfungus and Herbarium Collection. *P. irregulare* was obtained from the American Type Culture Collection. *Sordaria spp.* was obtained from the Carolina Biological Supply Company. *Pythium spp.* and *P. vexans* were obtained from the Department of Botany and Plant Pathology at Purdue University, West Lafayette. *A. sympodiale*, *Kretzschmaria* spp., *Pythium* spp., *P. irregulare*, *P. vexans*, and *Sordaria* spp. were grown on Potato Dextrose Agar (PDA), whereas, *A. muscivora* was grown on Cereal Agar (Table 1). PDA was made by mixing 4.0g of potato starch, 20.0g of dextrose (glucose), 15.0g of agar, and distilled water to 1.0L. This medium was brought to a boil in the microwave and autoclaved. Cereal Agar was made by mixing 25.0g of mixed cereal baby food (Gerber®), 5.0g of agar, and distilled H_2O to 250mL. This medium was brought to a boil in the microwave and autoclaved. Both PDA and

Cereal Agar were poured into petri dishes. All fungi were grown at room temperature (25°C) and did not receive direct light. The actively growing fronts of all specimens were used for experimentation. Using flame sterilized instruments and a laminar airflow hood, aseptic subculturing of the fungi was carried out every month or as needed for experimentation.

Cereal	25.0g Mixed Cereal Baby Food	5.0g Agar	250mL Distilled H_2O		
PDA	4.0g Potato Starch	20.0g Glucose	15.0g Agar	Distilled H_2O to 1.0L	
Solid LB	10.0g Tryptone	5.0g Yeast Extract	10.0g NaCl	7.5g Agar	Distilled H_2O to 1.0L
Liquid LB	10.0g Tryptone	5.0g Yeast Extract	10.0g NaCl	Distilled H_2O to 1.0L	
BCD	10.0mL Stock Solution B (MSO_4•$7H_2O$ 25.0g, distilled H_2O to 1L, autoclave, store in refrigerator)	10.0mL Stock Solution C (KH_2PO_4 25.0g, H_2O water to 500mL, adjust to pH 6.5 with KOH, autoclave, store in refrigerator)	10.0mL Stock Solution D (KNO_3 1.01g, $FeSO_4$•$7H_2O$ 1.25g, distilled H_2O to 1L, autoclave, store in refrigerator)	8.0g Agar	H_2O to 1.0L, pH to 6.5 with KOH

Table 1. The recipes for various media that were used to grow fungi and *A. serpens*.

ECC 7, 71, and P. aeruginosa Experimentation

Potato

100μL of ECC 7 and 71 were applied to pieces of *Solanum tuberosum* (potato). Qualitative results were recorded after 48 hours at 25°C.

A. serpens Protonema and Gametophyte

4μL of ECC 7, 71, and *P. aeruginosa* solutions were applied to uninjured and injured *A. serpens* protonema and gametophyte cultures kept at 25°C. LB medium without bacteria was applied as a control. *A. serpens* was injured by sterilizing scissors and cutting the moss immediately before or 12 hours before bacterial inoculation. Qualitative results were noted 1 week after inoculation.

Marchantia spp.

100μL of ECC 7, 71, and *P. aeruginosa* solutions were applied to uninjured and injured *Marchantia* spp. cultures grown at 25°C with 16 hours of light. LB medium without bacteria was applied as a control. *Marchantia* spp. was injured by sterilizing scissors and cutting the plant immediately before or 12 hours before bacterial inoculation. Qualitative results were noted 1 week after inoculation.

Fungi Experimentation

1cm^2 pieces of fungal growing fronts (*A. sympodiale, A. muscivora*, *Kretzschmaria* spp., *P. irregulare, Sordaria* spp., *Pythium* spp., and *P. vexans*) were applied next to two *A. serpens* specimen growing tips. Qualitative results of moss cell death, including cell browning and shriveling, were noted after 1 week.

A. serpens and P. irregulare Experimentation

Optimal Temperature for Primary Infection

1mm^2 pieces of PDA containing *P. irregulare* growing fronts were placed next to 24 *A. serpens* stem tips. Eight specimens were placed in an incubator at 20°C, eight specimens were kept at room temperature (25°C), and eight specimens were placed in an incubator at 30°C. After 24 hours, a 3mm piece of *A. serpens* growing tip was cut from each specimen and stained with 0.01% lacto-phenol trypan blue (1:1:1 lactic acid, phenol, water) for 30 seconds. A Leica (Wetzlar, Germany) DMLB Microscope with an attached SPOT imaging system (Diagnostic Instruments, Sterling Heights, Michigan, USA) was used to count and record appressoria locations (stem, leaves, or off moss) on *A. serpens*.

Hypersensitive Response

1mm^2 pieces of PDA containing *P. irregulare* growing fronts were placed next to *A. serpens* growing tips at room temperature (25°C). After 24 hours, 3mm pieces of moss growing tips were cut off. *A. serpens* cell death around fungal appressoria sites was determined by light microscopy. Cell death counts were carried out using a Leica (Wetzlar, Germany) DMLB Microscope with an attached SPOT imaging system (Diagnostic Instruments, Sterling Heights, Michigan, USA).

Systemic Acquired Resistance

First, a primary inoculation was completed. Pieces of PDA (1mm^2) containing *P. irregulare* growing fronts were placed next to *A. serpens* specimens at room temperature (25°C). After 24 hours, 3mm pieces of *A.* serpens growing tips were cut off, stained with 0.01% lacto-phenol trypan blue for 30 seconds, and rinsed with water. Appressoria and location on *A. serpens* (stem, leaves, or off moss) were counted using a Leica (Wetzlar,

Germany) DMLB Microscope with an attached SPOT imaging system (Diagnostic Instruments, Sterling Heights, Michigan, USA). Subsequently, more *A. serpens* pieces were removed and placed on BCD plates for 24 hours. A re-infection experiment was completed using *A. serpens* pieces free of fungus from the primary infection experiment. The same inoculation, staining, and counting procedures of the primary infection were followed.

Salicylic Acid Application

First, a stock solution of salicylic acid was made by dissolving 0.3478g of salicylic acid in 25mL of 95% ethanol. Then, 25, 50, 100, 500, and 1000µM solutions of salicylic acid were prepared using the stock solution (Table 2). Each dilution was prepared in a conical tube.

25µM	50µM	100µM	500µM	1000µM
10mL sterile H_2O	10mL	10mL	10mL	10mL
0.025mL stock solution	0.05mL	0.1mL	0.5mL	1.0mL

Table 2. Salicylic acid dilutions using a stock solution of salicylic acid dissolved in 95% ethanol.

Next, 1mL of each dilution was sprayed onto each culture of *A. serpens*. A total of eight moss specimens were used for each dilution and the sterile water control (48 total). The spraying apparatus consisted of a plastic hose connected at one end to an air nozzle and the other end to a sterile Pasteur pipette. Another sterile Pasteur pipette was put, narrow end up, in the salicylic acid dilution. With the air on, the narrow ends of the Pasteur pipettes were put close together. The conical tube measurements allowed spraying volumes to be monitored (Fig. 6).

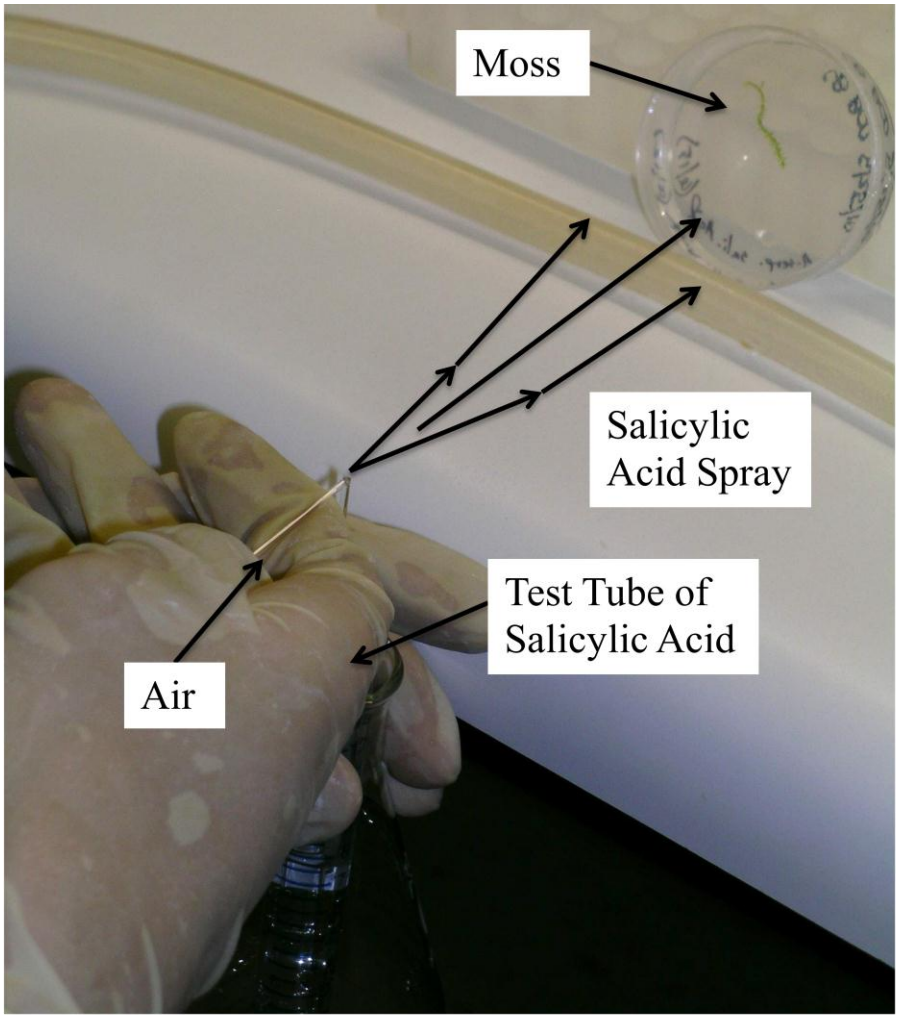

Figure 6. Apparatus used to spray salicylic acid onto *A. serpens* specimens.

After 24 hours, each moss specimen was inoculated with *P. irregulare*. The previously stated inoculation procedure was followed. A 3mm section of each moss specimen was stained with lacto-phenol trypan blue following the above stated procedure. *P. irregulare* appressoria were counted using a Leica (Wetzlar, Germany) DMLB Microscope with an attached SPOT imaging system (Diagnostic Instruments, Sterling Heights, Michigan, USA).

Protein Extraction

Stock solutions for *A. serpens* protein extraction were made. This included a stock extraction buffer, running buffer, 1M tris(hydroxymethyl)aminomethane (TRIS) at pH 7.5 and 8.3, a stock sample buffer, multiple destains, Coomassie blue stock solution, stain for the gel, and BSA standards (Table 3). Bovine serum albumin (BSA) standards (200, 400, 600, and 800μg/mL) were made by adding 5mL of Bradford reagent to 100μL of each BSA concentration. A spectrophotometer set to 575nm was used to determine absorbency readings. 1mL of 500μM SA was sprayed onto six moss specimens. Every 20 minutes for 2 hours, 0.8-0.12 grams of moss from one specimen was frozen in liquid nitrogen and ground-up with a mortar and pestle. 1mL of extraction buffer was added. The liquid was put into an Eppendorf tube and centrifuged for 3 minutes. 5mL of Bradford reagent was added to 200μL of supernatant. To determine protein concentrations, absorbency readings for moss specimens were compared with the BSA standard absorbencies. 10μg of protein from each 20 minute time point were loaded into a 4-15% polyacrylamide gradient gel with a loading dye and water to 30μL. The gel was run for 1 hour and 45 minutes at 125 volts. It was stained with a Coomassie blue stain for 1 hour. Both destain 1 and 2 were applied for 30 minutes until bands could be seen.

Gel Stain	250mL Methanol	50mL Acetic Acid	50mL Stock Stain	50mL H_2O
Coomassie Blue Stain	0.5g Coomassie Blue	50mL H_2O		
Destain 2 (400mL)	28mL 7% Acetic Acid	20mL 5% Methanol	352mL H_2O	
Destain 1 (250mL)	125mL 50% Methanol	25mL 10% Acetic Acid	100mL H_2O	
Sample Buffer	10.0g Glycerol	20.0mL TRIS-HCl pH 6.8	1.0g SDS	2mL 10% Bromophenol Blue
Running Buffer	6.0g TRIS	28.8g Glycine	20.0mL SDS	
Extraction Buffer	12.1g TRIS	37.2g EDTA	10.0g SDS	

Table 3. The recipes for various solutions and stains used in the protein extraction procedure.

A. serpens Chemical Extraction and Identification

Five mosses were sprayed with 100 and 1000μM SA. Moss without SA was used as a control. A supercritical CO_2 chemical extraction method was used. Whole moss specimens (approximately 0.5 grams) were placed in conical tubes and covered with solid CO_2 (dry ice). The conical tubes were capped and placed in warm water until the solid CO_2 turned to liquid and then evaporated. Moss specimens were removed. 1mL of dichloromethane was used to dissolve the extractant. The extractant was subjected to gas chromatography-mass spectrometry (GC-MS) using a Varian 220 GC-MS outfitted with a non-polar column.

Statistics

Excel 2007 was used to calculate means and standard errors. Minitab was used to calculate Mann-Whitney, Kruskal-Wallace, Wilcoxon sign rank, paired difference, and Tukey's multiple comparison tests.

RESULTS

A survey of pathogenic bacteria and fungi

Initially, pathogenic bacteria with a wide host range, were investigated for their ability to infect various non-vascular plants. First, ECC 7 and 71 did not infect uninjured *A. serpens* protonemata or gametophytes, *C. purpureus*, or *Marchantia* spp. (a liverwort). Significant ECC 7 and 71 populations had grown successfully *in vitro* because the medium was turbid. Positive signs of infection include chloroplast and stem browning, chloroplast , and cellular necrosis. However, after 4μL applications of bacteria (excess amounts), no visual signs of infection were present after 1, 2 and 7 days. Second, ECC 7

and ECC 71 did not infect injured *A. serpens* protonemata or gametophytes, *Marchantia* spp., or *C. purpureus*. Scissors or pins were used to mechanically injure the non-vascular plant specimens. Both immediate and 24 hours after injury bacterial applications showed no visual signs of infection after 1, 2, or 7 days.

A. muscivora, Kreezschmaria spp., and P. irregulare cause cell death in A. serpens

A. serpens was exposed to *A. sympodiale, A. muscivora, Kreezschmaria* spp., *Pythium* spp., *P. irregulare,* and *P. vexans* (Fig. 7). Seven days after exposure, *P. irregulare* grew rapidly to cover *A. serpens* (Fig. 8). Visual signs of *A. serpens* death including chloroplast and stem browning, chloroplast degradation, and cellular necrosis were noted with application of *A. muscivora, Kreezschmaria* spp., and *P. irregulare*. After seven days, complete moss death did not occur. Cell death seemed to be confined to particular regions (Fig. 9). *P. irregulare* exposure caused the greatest degree of moss cell death in the shortest amount of time. Thus, *P. irregulare* was chosen to work with.

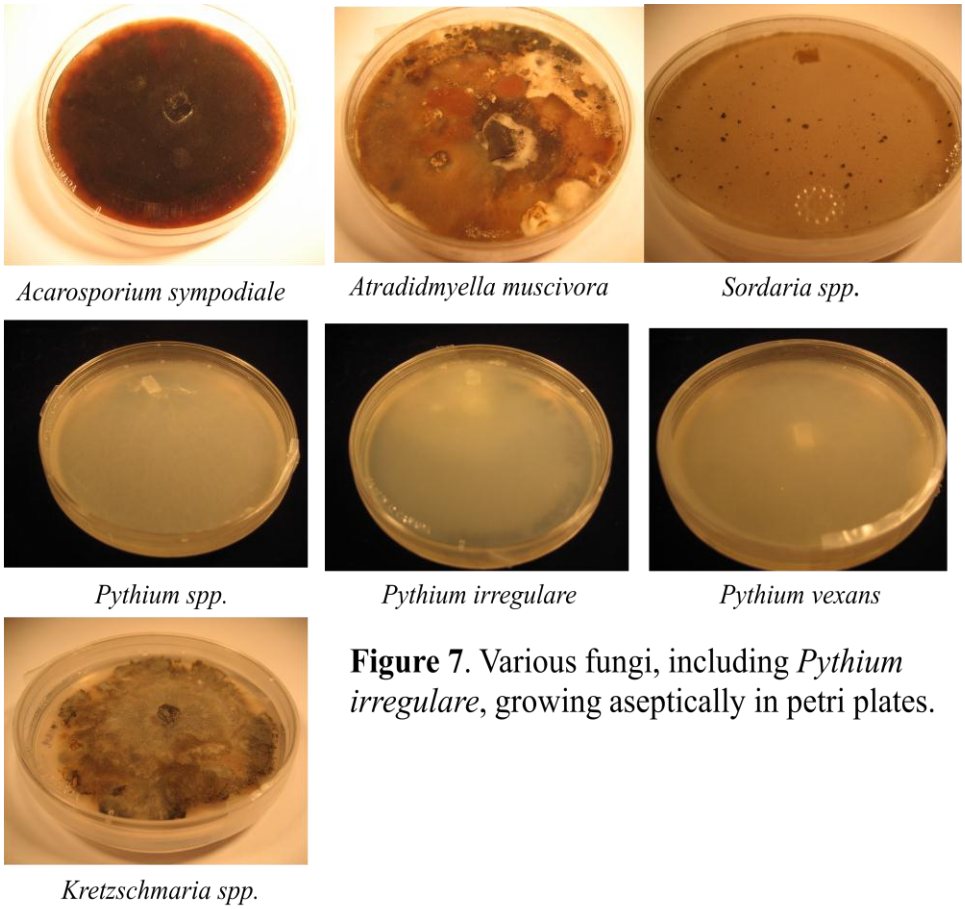

Figure 7. Various fungi, including *Pythium irregulare*, growing aseptically in petri plates.

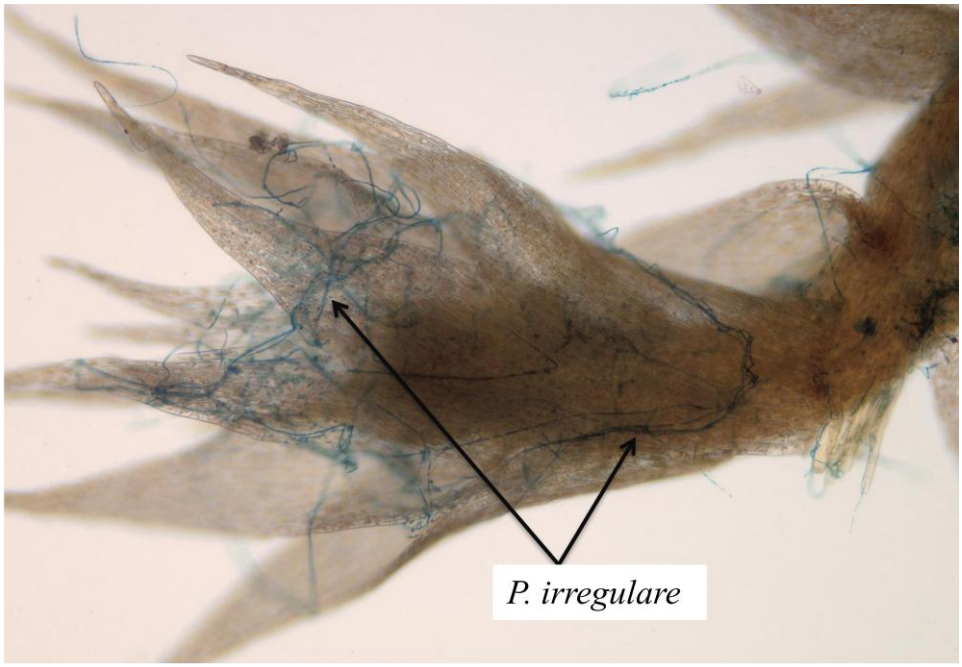

Figure 8. *A. serpens* gametophyte stem-tip covered with selectively stained *P. irregulare*.

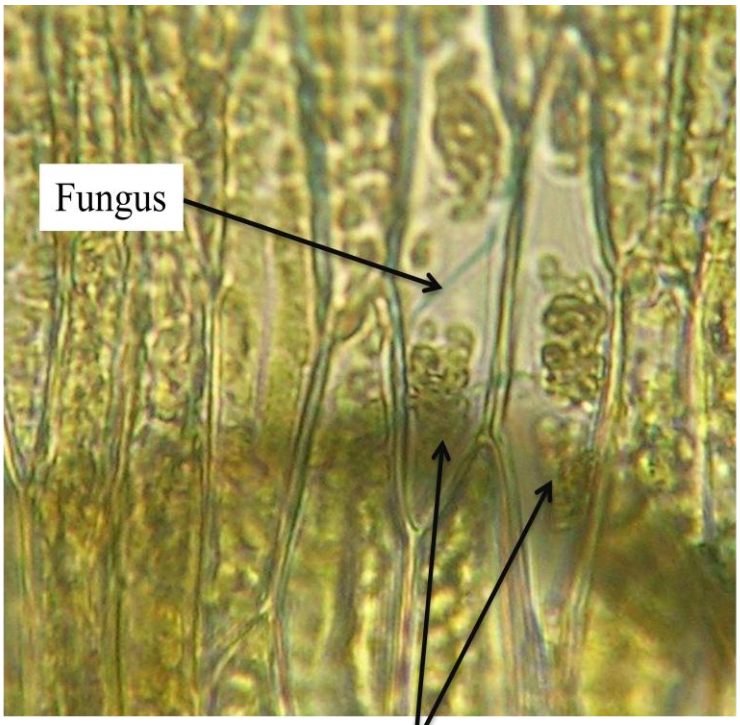

Figure 9. Chloroplast degradation in *A. serpens* cells infected with *P. irregulare*.

P. irregulare infects A. serpens

Utilizing a differential lacto-phenol trypan blue stain and light microscopy, *P. irregulare* appressoria were investigated. A small number of appressoria penetrated the cell wall of *A. serpens* cells (Fig. 10). A darker blue colored infection peg was inside the fluid environment of the appressorium. Due to hydrostatic pressure and cellulose digesting enzymes, the infection peg can be seen penetrating the cell wall of *A. serpens*. Moreover, hyphal growth inside the moss cell was seen. This growth did not stain blue because lacto-phenol trypan blue does not penetrate the moss cell wall.

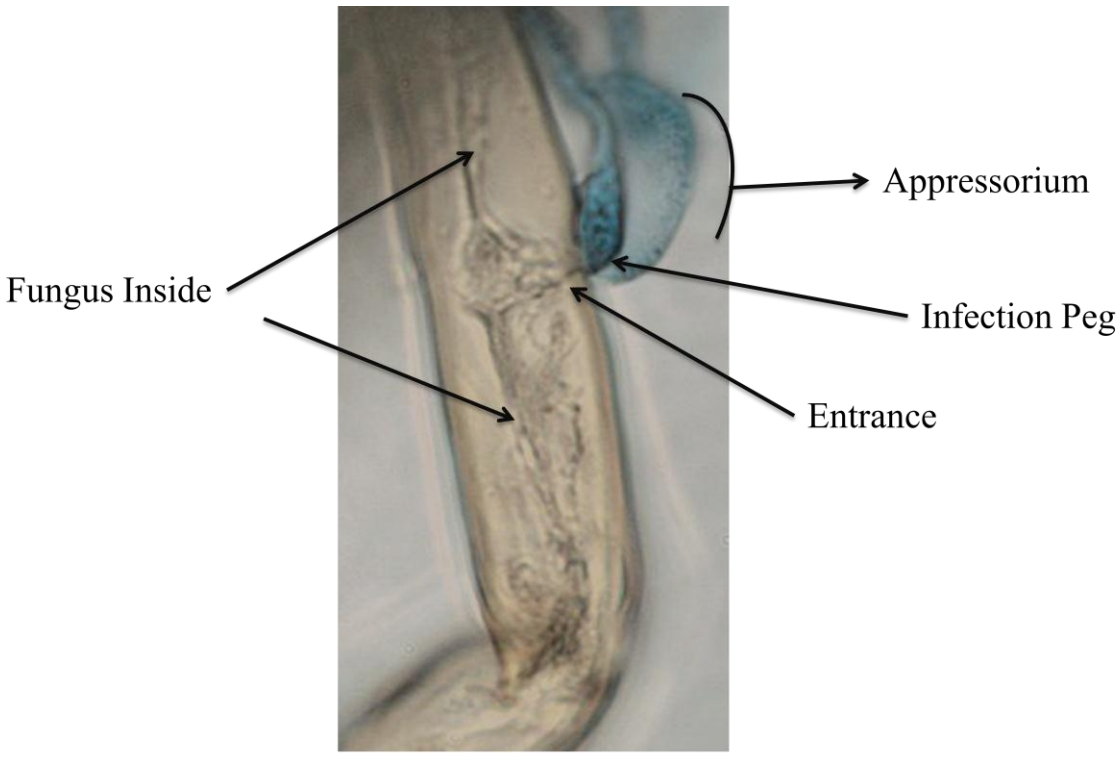

Figure 10. *Pythium irregulare* appressorium infection site on *Amblystegium serpens*.

The optimum temperature for appressoria formation is room temperature (25°C) and most appressoria were found on moss leaves

The number of appressoria were counted on 3mm pieces of *A. serpens* stem tips exposed to *P. irregulare* at 20, 25, and 30°C. Room temperature (25°C) had the most appressoria (Fig. 11a). Specifically, 25°C had a mean of 20 appressoria (n=8), 30°C had a mean of 15.5 appressoria (n=8), and 20°C had the lowest mean with 6.125 appressoria (n=8). A Kruskal-Wallis statistical test was completed. Although there was not a significant difference, temperature did appear to influence appressoria formation (P = 0.175).

Locations of appressoria on 3mm *A. serpens* pieces at 25°C were also noted. Most appressoria were found on *A. serpens* leaves. A mean of 9.278 appressoria were on leaves (n=8), 5.929 appressoria were on stems (n=8), and 3.1 appressoria were not on *A.*

serpens (n=8)(Fig. 11b). A Mann-Whitney statistical test was completed. Although there was not a significant difference, temperature did appear to influence appressoria location (P = 0.118). Most likely, these appressoria became detached during the movement and staining of the moss pieces.

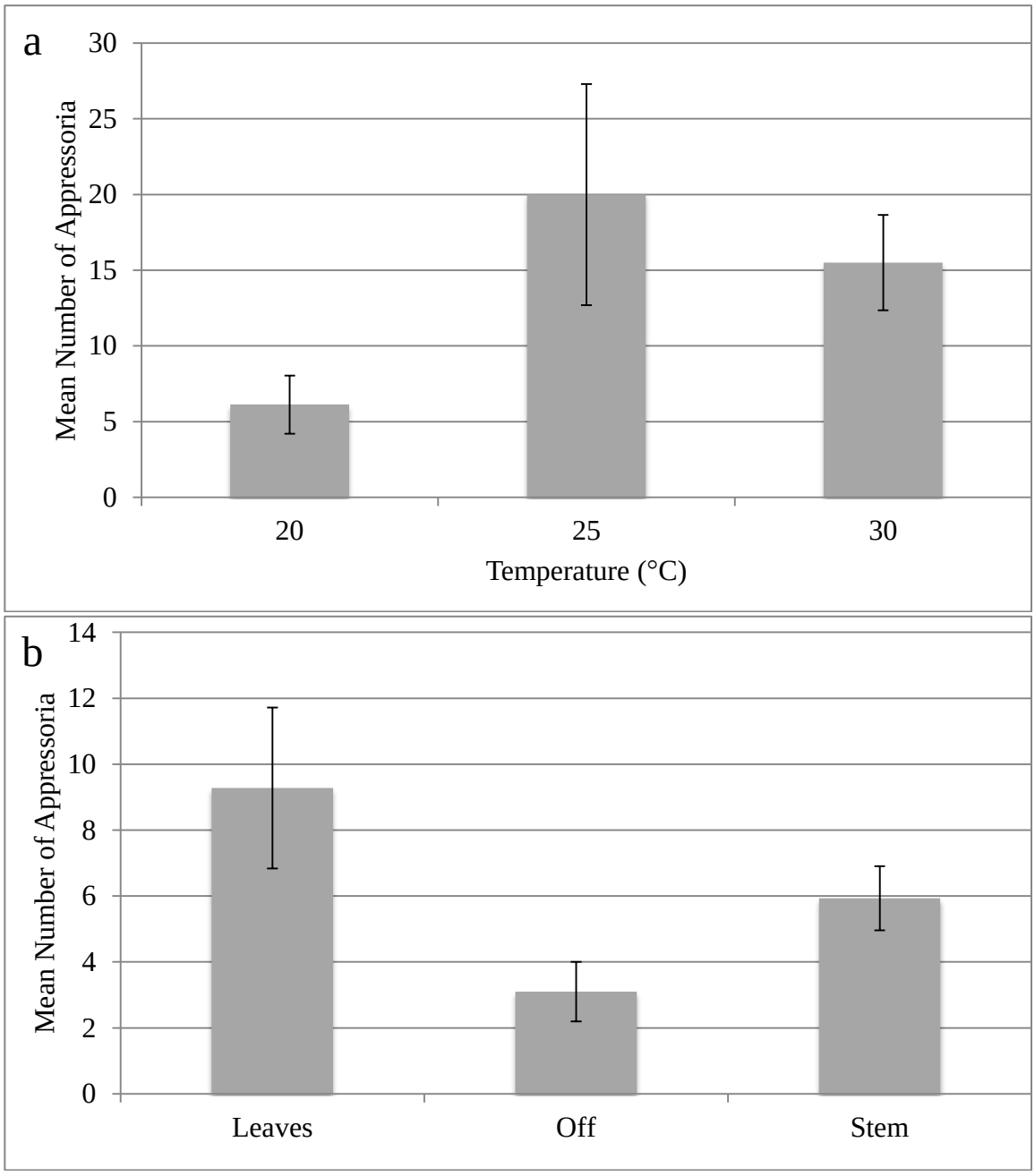

Figure 11. The influence of temperature on appressoria formation and location. 1mm square pieces of *P. irregulare* medium were placed next to *A. serpens* stem tips for 48 in the dark at 20°C, room temperature (25°C), and 30°C. Eight *A. serpens* cultures were used for each temperature. **a.** Appressoria for each *A. serpens* specimen were counted on a 3mm piece of stem tip. **b.** Number of appressoria in different locations at 25°C. Means and standard errors were calculated for each temperature. A Kruskal-Wallis statistical tests was completed for the temperature experiment (P = 0.175). A Mann-Whitney test was completed for the location experiment (P = 0.118).

P. irregulare appressoria increased 48 hours after exposure to A. serpens

Twenty-four hours after *P. irregulare* exposure, there was a mean of 6.54 appressoria on the 3mm piece of *A. serpens* stem tip (Fig. 12). Forty-eight hours after *P. irregulare* exposure, there was an increase of appressoria to a mean of 20.08. This was a 67.4% increase in appressoria from day 1 to day 2.

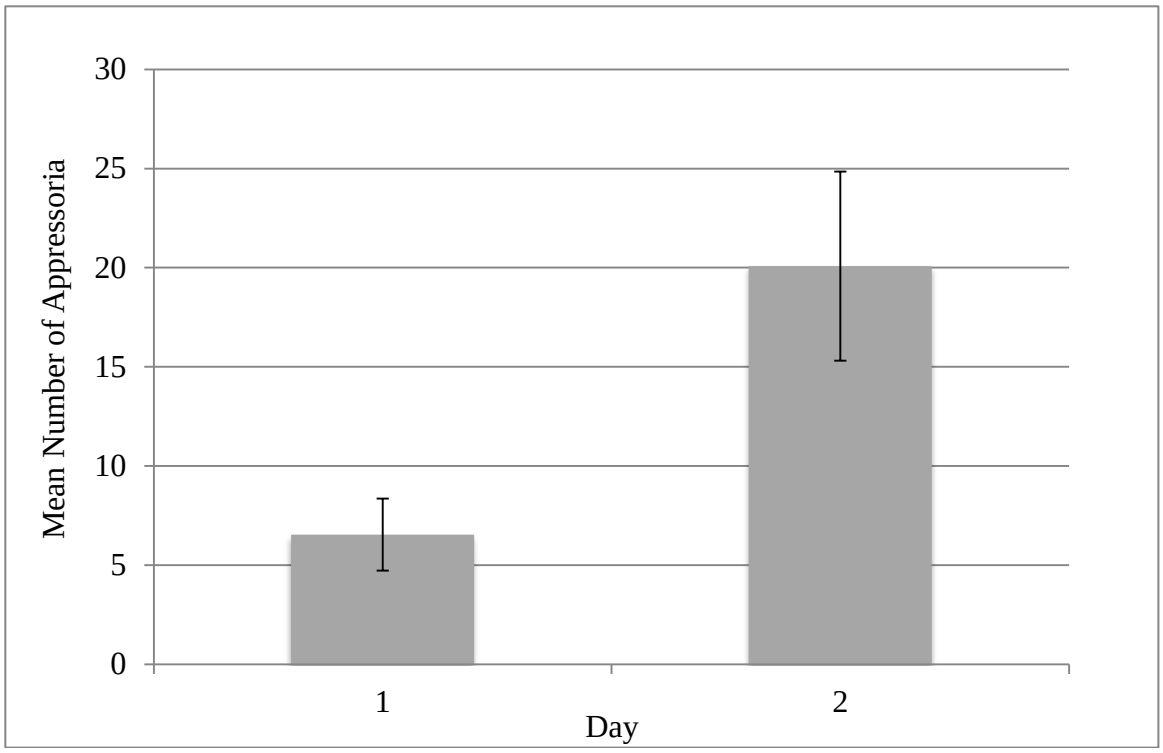

Figure 12. The number of appressoria present 1 or 2 days after primary exposure. 1mm square pieces of *P. irregulare* medium were placed next to *A. serpens* stem tips for 48 at room temperature (25°C). Four *A. serpens* cultures were used for each day. Every 24 hours, the number of appressoria for each *A. serpens* culture were counted on a 3mm piece of stem tip. Seven out of the eight *A. serpens* cultures had appressoria on them. Means and standard errors were calculated for each day.

Number of Pythium irregulare appressoria after re-exposure

P. irregulare primary exposure to *A. serpens* with subsequent re-exposure was undertaken to determine if defense responses by the moss would prevent appressoria formation. Appressoria were present on all of the nine primary exposure *A. serpens* specimens (Fig. 13). Appressoria were present on 86% (6 out of 7) of the re-exposure *A. serpens* specimens; 3 specimens were contaminated and not included.

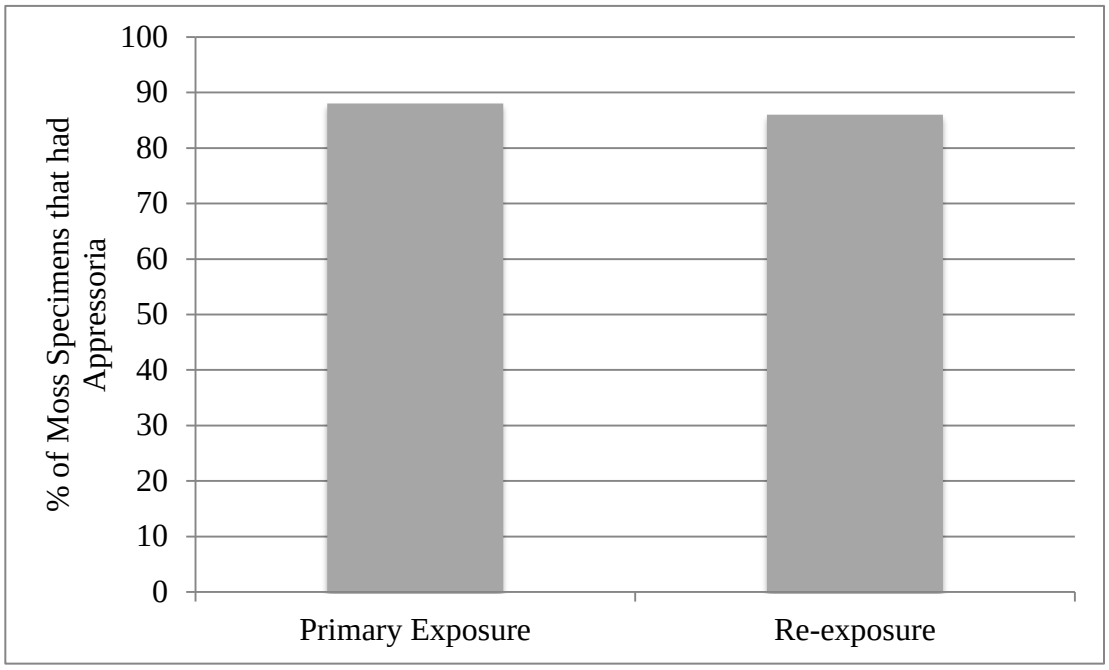

Figure 13. Percentage of *A. serpens* cultures with appressoria present. 1mm square pieces of *P. irregulare* medium were placed next to *A. serpens* stem tips for 24 hours at room temperature (25°C) in the dark. Ten *A. serpens* cultures were used. *A. serpens* pieces were cut, removed, and placed on new medium for 24 hours. Re-exposure was then carried out. Appressoria on each *A. serpens* re-exposure specimen were counted from a 3mm piece of stem tip.

P. irregulare primary exposure had a mean of 7.333 appressoria on the 3mm pieces of *A. serpens* stem tip with most appressoria found on the leaves of *A. serpens* (n=8) (Fig. 14). Examination of the location of the appressoria after primary exposure showed a mean of 6.0 appressoria were present on the leaves (n=10), 2.6 appressoria were present on the stems (n=10), and 1.571 appressoria were not found on the moss (n=10) (Fig. 15). A Mann-Whitney statistical test was completed. Although there was not a significant difference, the location of appressoria may be different in the primary exposure (P = 0.125).

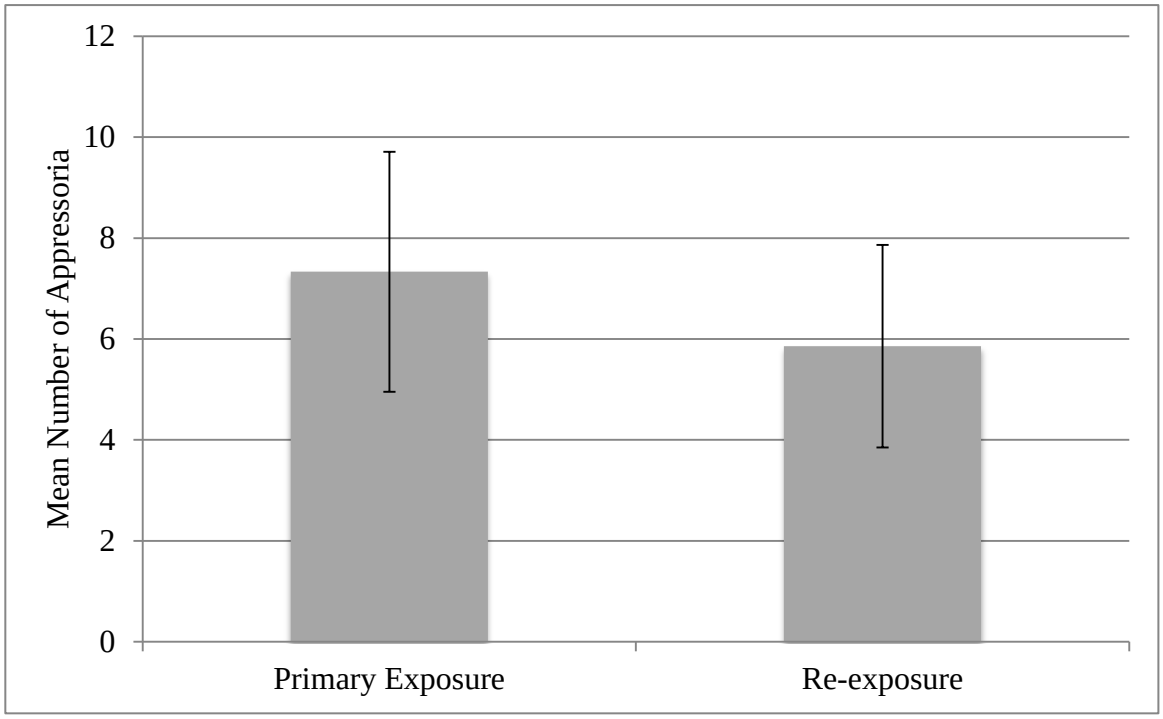

Figure 14. Number of appressoria after primary exposure and re-exposure . 1mm square pieces of *P. irregulare* medium were placed next to *A. serpens* stem tips for 24 hours at room temperature (25°C) in the dark. Eight *A. serpens* cultures were used. After one day, fungus-free *A. serpens* pieces were cut, removed, and placed on new medium for 24 hours. Re-exposure was then carried out. Appressoria on each *A. serpens* re-exposure specimen were counted from a 3mm piece of stem tip. Means and standard errors were calculated. A Wilcox signed-rank statistical test was completed.

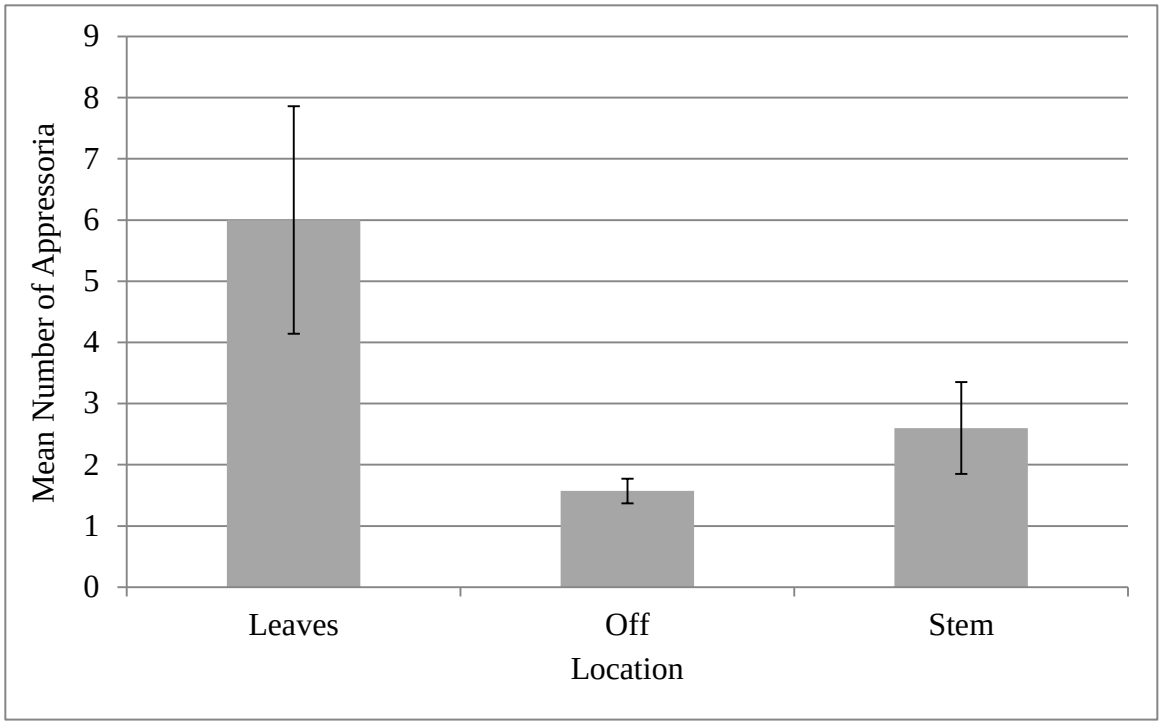

Figure 15. From the primary exposure experiment, appressoria locations (leaves, stem, or off moss) with primary exposure of *P. irregulare*. 1mm square pieces of *P. irregulare* medium were placed next to *A. serpens* stem tips for 48 in the dark at room temperature (25°C). Ten *A. serpens* cultures were used. Appressoria locations for each *A. serpens* specimen were counted on a 3mm piece of stem tip. Means and standard errors were calculated for each location. A Mann-Whitney statistical test was completed (P = 0.125).

When I examined *A. serpens* after re-exposure, I found *P. irregulare* re-exposure had a smaller mean with 5.857 appressoria (Fig. 14). Again, most were found on the leaves of *A. serpens*. A Wilcoxon-Signed Rank statistical test was completed. There was no statistical difference between the treatments (T = 13; Tcrit. = 2). A mean of 4.833 appressoria were on the leaves, 4.0 were on the stems, and 1.333 were off the moss (Fig. 16). A Mann-Whitney statistical test was completed. There was a significant difference in location of appressoria with the re-exposure experiment (P = 0.045).

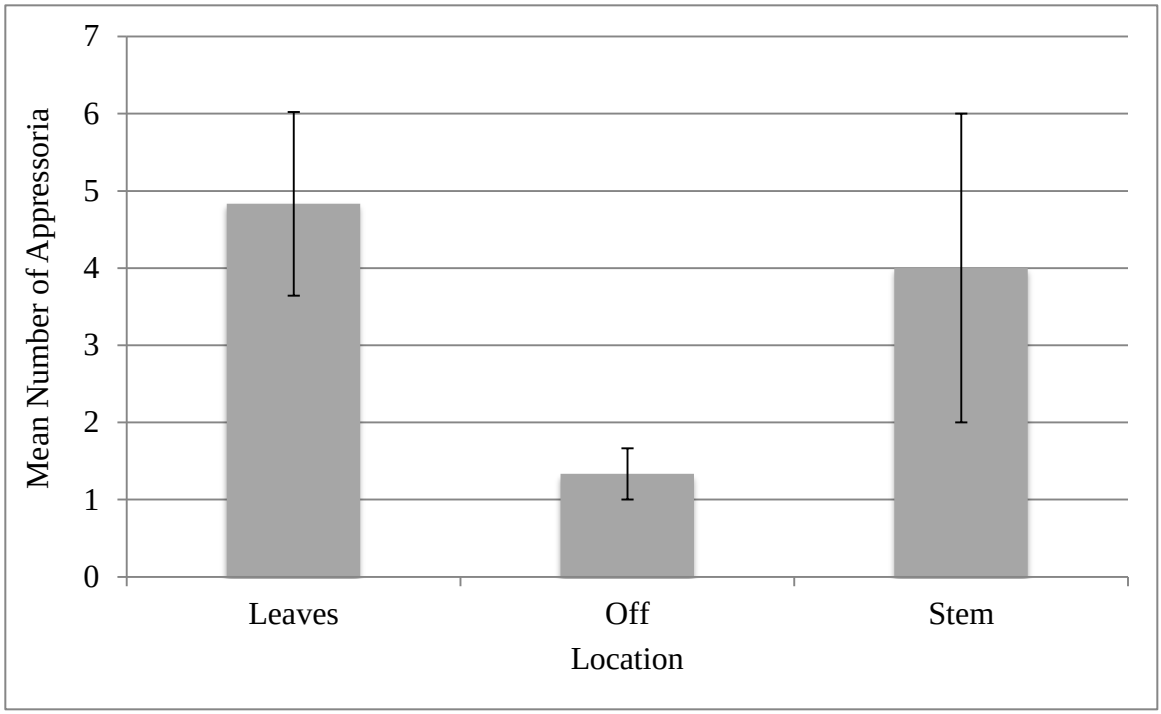

Figure 16. From the re-exposure experiment, appressoria locations (leaves, stem, or off moss) with re-exposure of *P. irregulare*. 1mm square pieces of *P. irregulare* medium were placed next to *A. serpens* stem tips for 48 in the dark at room temperature (25°C). Ten *A. serpens* cultures were used. Appressoria locations for each *A. serpens* specimen were counted on a 3mm piece of stem tip. Means and standard errors were calculated for each location. A Mann-Whitney statistical test was completed (P = 0.045).

The mean number of appressoria penetrations of A. serpens cells decreased with re-exposure of P. irregulare

P. irregulare was exposed to seven *A. serpens* cultures for 3 days. Fifteen randomly picked appressoria were assessed for *A. serpens* cell penetration. The minimum number of penetrated cells was 1, and the maximum was 4 (Fig. 17). A mean of 2.57 *A. serpens* cells were penetrated by appressoria (Fig. 18). A re-exposure of *P. irregulare* to *A. serpens* pieces not covered with fungus was then undertaken. One specimen did not have any penetrated cells, but the others had 1 to 4 penetrated cells (Fig. 17). A mean of 1.71 *A. serpens* cells were penetrated by appressoria, a 33.5% decrease (Fig. 18). Not enough samples were present to complete statistical tests.

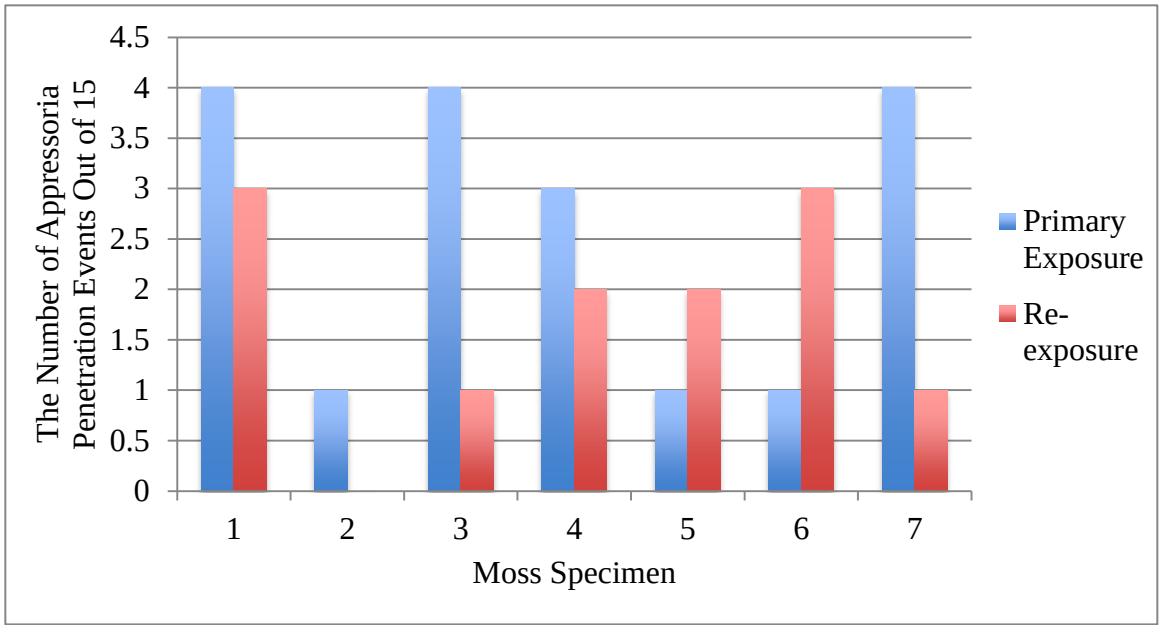

Figure 17. *P. irregulare* appressoria penetration into *A. serpens* leaf cells. 1mm square pieces of *P. irregulare* medium were placed next to seven *A.* serpens cultures. After 3 days, a 3mm piece of each *A. serpens* culture was taken and stained with lacto-phenol trypan blue. Using a Leica DMLB Microscope with an attached SPOT imaging system, 15 appressoria were randomly counted and assessed for moss cell penetration. *A. serpens* fungus-free pieces were cut, removed, and placed on new medium for 24 hours. Re-exposure and cell penetration counts were then carried out. The seven *P. irregulare* cultures with penetration values are shown.

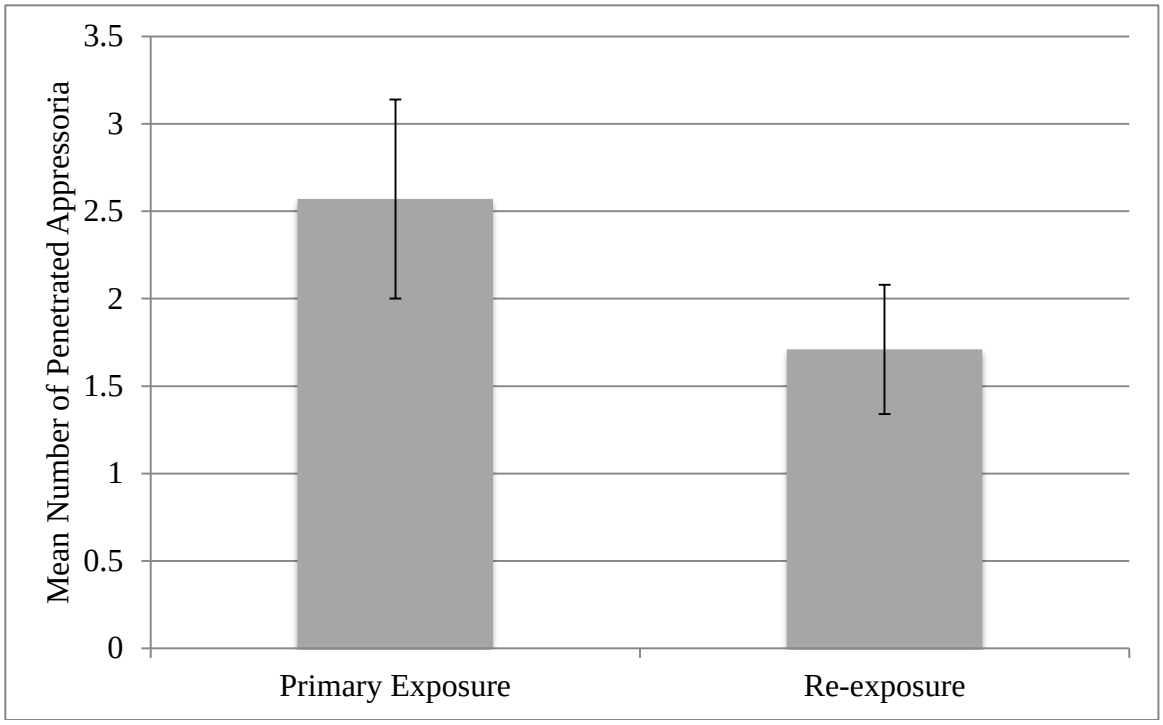

Figure 18. *P. irregulare* appressoria penetration into *A. serpens* cells. 1mm square pieces of *P. irregulare* infused medium were placed next to seven *A.* serpens cultures. After 3 days, a 3mm piece of each *A. serpens* culture was taken and stained with lacto-phenol trypan blue. Using a Leica DMLB Microscope with an attached SPOT imaging system, 15 appressoria were randomly counted and assessed for moss cell penetration. *A. serpens* pieces were removed and placed on new medium for 24 hours. Re-exposure and cell penetration counts were then carried out. Means and standard errors were calculated for the primary exposure and re-exposure experiments. A Wilcoxon Signed-Rank statistical test was completed.

SA does not influence A. serpens or P. irregulare growth

SA concentrations of 25, 50, 100, 500, and 1000µM were sprayed onto *A. serpens* cultures and mean stem tip growths were measured after 7 days (Fig. 19). The moss control mean growth was 6.503mm, the moss and water grew 4.225mm, and the moss and *P. irregulare* exposure grew 3.582mm. The SA concentrations of 25, 50, 100, 500, and 1000µM were 6.597, 5.366, 3.382, 3.674, and 5.917mm. There was a statistical difference between 25µM SA treatment and 100µM SA and the moss *P. irregulare* treatments ($F = 2.89$; $P = 0.01$).

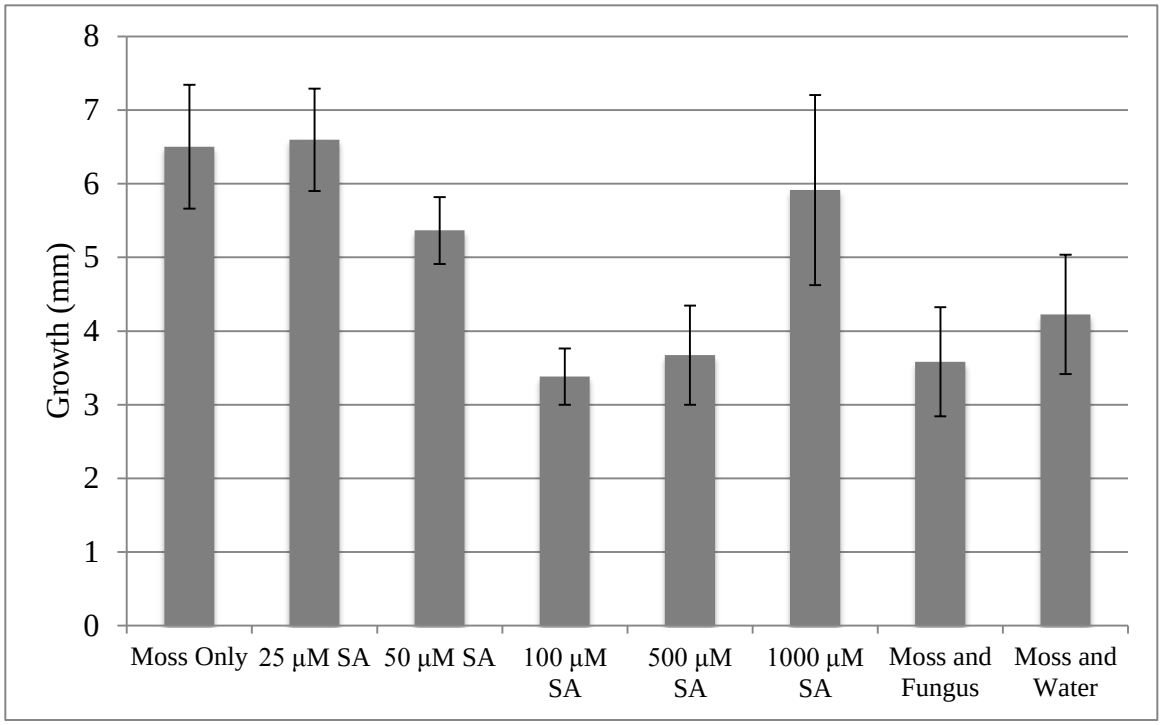

Figure 19. The influence of salicylic acid treatments and *P. irregulare* exposures on *A. serpens* growth. Salicylic acid and water were sprayed (1mL) on individual *A. serpens* cultures. 1mm square pieces of *P. irregulare* medium were placed next to *A.* serpens in only the moss with *P. irregulare* treatment. After 7 days, the growth (mm) of all stem tips were measured with Motic Image Plus 2.0 ML. Three cultures were used for each treatment. Means and standard errors were calculated for each treatment. ANOVA and Tukey's statistical tests were completed. See text for explanation of statistics.

SA concentrations of 500 and 1000µM were also sprayed onto *P. irregulare* and mean hyphal growths were measured after 24 hours (Fig. 20). The *P. irregulare* control grew 11.505mm, and the *P. irregulare* with water grew 13.237mm. *P. irregulare* with SA treatments grew similarly to the controls. *P. irregulare* with 500 and 1000µM SA grew 12.195mm and 12.488mm.

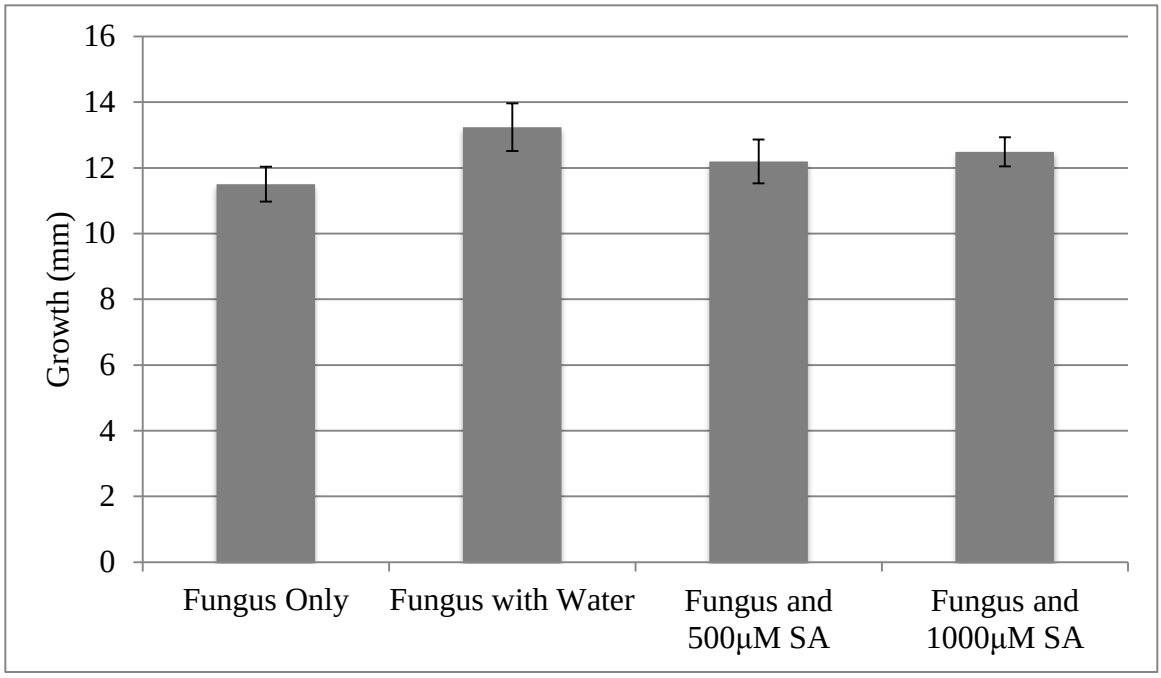

Figure 20. The influence of salicylic acid treatments on *P. irregulare* growth. Salicylic acid and water were sprayed (1mL) on individual *P. irregulare* sub-cultures. After 24 hours, the growth (mm) of the growing fronts were measured with a Motic Image Plus 2.0 ML. Means and standard errors were calculated for the three *P. irregulare* sub-cultures used in each treatment. ANOVA and Tukey's statistical tests were completed. No statistical difference was noted.

SA concentrations of 25, 100, and 1000µM had an undetermined influence on P. irregulare appressoria penetration events on A. serpens leaf cells

1mL of 25, 100, and 1000µM SA was sprayed onto *A. serpens* cultures. Three hours later, *P. irregulare* was exposed to the moss and moss cell penetrations were counted after 24 hours of exposure (Fig. 21). A consistent decrease in appressoria penetration events occurred with the increasing SA concentrations. *A. serpens* with 25µM SA had a mean of 3.6 penetrations (n=5), 100µM had 2.8 penetrations (n=5), and 1000µM had 1.8 penetrations (n=5). However, the control did not support this. *A. serpens* sprayed with 1mL of H_2O had 1.4 penetrations. A Kruskal-Wallace statistical test was completed. No difference between treatments was noted. Larger sample sizes should be tested in the future.

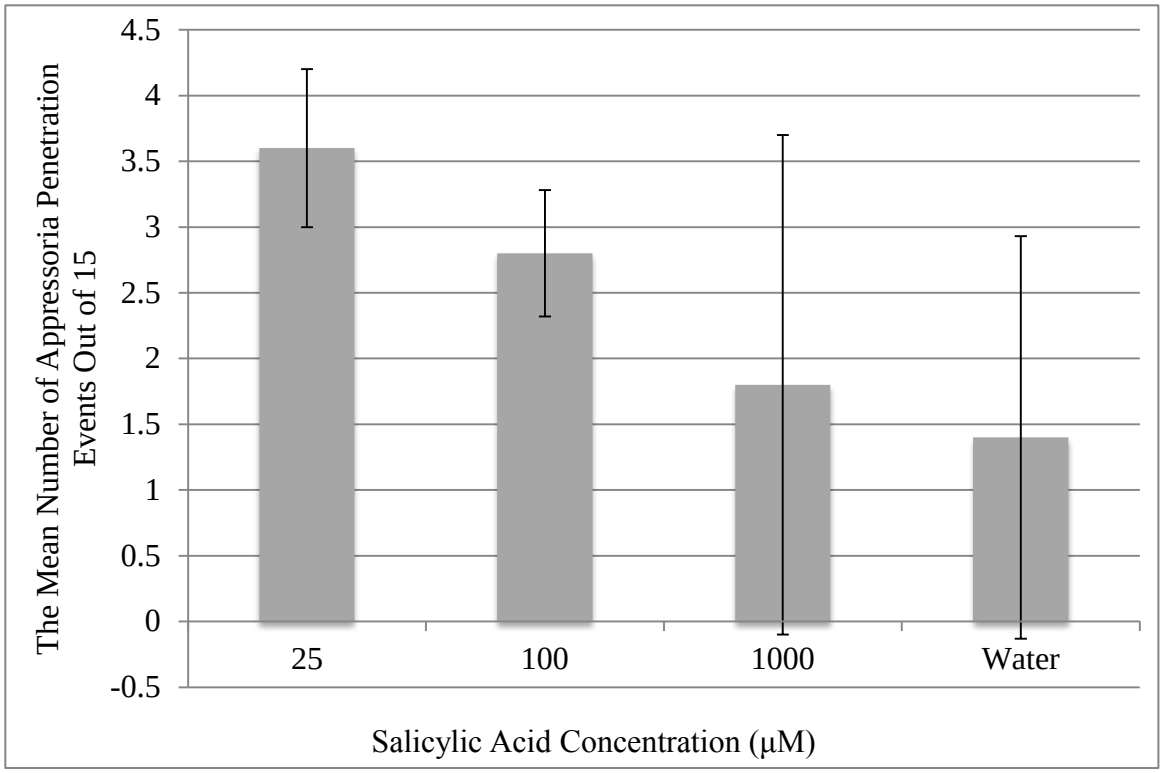

Figure 21. *P. irregulare* appressoria penetration into *A. serpens* leaf cells. 1mL of SA was sprayed onto *A. serpens* cultures (5 for each treatment). Four hours later, 1mm square pieces of *P. irregulare* infused medium were placed next the *A.* serpens cultures. After 24 hours, a 3mm piece of each *A. serpens* culture was taken and stained with lacto-phenol trypan blue. Using a Leica DMLB Microscope with an attached SPOT imaging system, 15 appressoria were randomly counted and assessed for moss cell penetration. The means and standard errors are shown. A Kruskal-Wallace statistical test was completed. No statistical difference was noted.

A. serpens chemical and protein profile identifications were unsuccessful

An attempt was made to compare chemical and protein profiles before and after SA treatment. Chemical extraction using supercritical CO_2 and subsequent chemical identification using gas chromatography-mass spectrometry (GC-MS) were not successful. No chemicals were present on the GC-MS readout. Protein profiling was also undertaken. Proteins were isolated from *A. serpens*. After gel electrophoresis, proteins bands were heavy and smeared together. Identification of protein band differences before and after SA treatment was unsuccessful.

DISCUSSION

ECC 7, 71, and P. aeruginosa do not infect A. serpens, C. purpureus, or Marchantia spp.

Bacterial inoculations of *A. serpens*, *C. purpureus*, or *Marchantia* spp. with ECC 7, 71, and *P. aeruginosa* were not effective. Signs of infection including chloroplast and stem browning, chloroplast degradation, and cellular necrosis were not seen with any of these non-vascular plants. This is interesting because these bacteria have wide plant host ranges. Ponce de León *et al.* (2007) demonstrated that ECC infects, causes severe cellular necrosis, and activates HR and SAR in the non-vascular plant *P. patens*. He (1996) demonstrated that *P. aeruginosa* has *hrp* genes that encode for cell-wall-degrading enzymes. Moreover, these *hrp* genes are widely conserved in the bacterial kingdom. Finally, Rahme *et al.* (2000) demonstrated that *P. aeruginosa* opportunistically infected *Arabidopsis thaliana* plants and lettuce leaves resulting in soft-rot symptoms including cell necrosis and midrib softening.

These bacteria are capable of infecting plants, but various aspects necessary for infection were not met within my experiment. First, ECC 7 and 71 are different strains than used by Ponce de León *et al.* (2007). All other experimental aspects were the same. These included growing the bacteria overnight in LB medium at 28°C, applying the bacteria in a similar manner, and waiting two days to examine for infection. Different strains could have different virulence mechanisms, such as dissimilar cell membrane receptors or lower production of cell-wall degrading enzymes. This could account for why ECC 7 and 71 did not infect any non-vascular plant I tried. Davey and Currah (2006) reiterate this point by showing that different fungi have different virulence mechanisms that allow only certain parts of the moss to be infected (*i.e.* only stem or

rhizoids). Second, Rahme *et al.* (2000) described how *P. aeruginosa* is an opportunistic pathogen in both humans and plants. An opportunistic pathogen is an organism that is only pathogenic when the host organism defenses are compromised. Thus, it makes sense that the uninjured non-vascular plants did not show infection symptoms. Moreover, injury to the non-vascular plants consisted of making a small cut or poking a small hole in the plant. It is possible that this level of injury did not sufficiently compromise the plant defenses in order for *P. aeruginosa* to infect. Overall, prior research has shown that the bacteria tested do infect various types of plants. Within my experiment, bacterial strain differences or insufficient moss injury could explain why infection was not noted.

A. muscivora, Kreezschmaria spp., and P. irregulare cause cell death in A. serpens

A. serpens chloroplast and stem browning, chloroplast degradation, and cellular necrosis were noted seven days after exposure to the fungi *A. muscivora, Kreezschmaria spp.,* and *P. irregulare*. *P. irregulare* exposure caused the greatest degree of moss cell death in the shortest amount of time. Regardless of exposure time, *A. serpens* specimens were never completely killed. The fungal infection seemed to be confined to particular regions. This highlights two things. First, the moss cell death suggests that these three fungi are capable of infecting *A. serpens*. Davey *et al.* (2009) support this assumption with *A. muscivora*. They showed that this fungus infects the moss *Funaria hygrometrica* and causes moss leaves to become brown and mottled. Cell wall degradation was also noted. Moreover, Oliver *et al.* (2009) and Ponce de León *et al.* (2007) showed that *P. irregulare* infects the non-vascular plant *P. patens,* and Adie *et al.* (2007) showed that *P. irregulare* infects *A. thaliana*. This resulted in stem browning, softening of moss tissues,

and hyphal growths throughout the intracellular spaces. Second, incomplete killing of moss specimens suggests that *A. serpens* has pathogen defenses perhaps HR and SAR. Since the moss never completely died, these defenses might be responsible for quarantining the pathogen to particular regions and preventing subsequent infections. To the best of my knowledge, prior research has not shown that *A. serpens* displays HR and SAR. On the whole, exposure of these three fungi to *A. serpens* cause visual signs of infection, but it is likely that moss defenses prevent the whole plant from being infected.

P. irregulare infects A. serpens

Although visual signs of infection within the moss are good indicators of fungal infection, microscopic evidence of *P. irregulare* infection had to be elucidated. First, chloroplast degradation and clustering in the corners of moss cells was abundantly noted (Fig. 9). In some instances, hyphal growth could be noted in areas devoid of chloroplasts Davey *et al.* (2009) published electron micrographs that are very similar to the micrographs within this report. Both show a mottled moss appearance due to chloroplast degradation, and individual moss cells showing signs of infection due to intracellular hyphal growths. These similarities reinforce the validity of my experimental methodology taken to infect *A. serpens* with *P. irregulare*.

However, Davey *et al.* (2009) supplemented light microscope micrographs with detailed electron microscope micrographs allowing appressoria and hyphal strands within moss cells to be clearly seen. Future research using the electron microscope to look at fungal hyphal strands and appressoria would greatly enhance our understanding of how *P. irregulare* infects *A. serpens*.

The optimum temperature for appressoria formation is room temperature (25°C) and most appressoria were found on moss leaves

In order to study infection, environmental factors that influence *P. irregulare* appressoria formation must be optimized. Temperature is one environmental parameter that was considered. Oliver *et al.* (2009) kept moss cultures at 22°C during *P. irregulare* exposure. Adie *et al.* (2007) kept their moss cultures at 21°C during *P. irregulare* exposure. Whereas this is not a large difference in temperature, my results show that ±5°C from 25°C has significant impacts on appressoria formation (Fig. 11a).

Why does temperature play an important role in appressoria formation? Plants cannot regulate their internal temperature, so they function at ambient temperature. This temperature dictates enzyme-catalyzed reaction rates within plant cells. Many enzymes are important for metabolic reactions to occur. Higher temperatures will increase the kinetic energy of the enzymes and substrates. This increases the number of collisions between the enzymes and substrates, which increases the enzyme-catalyzed reaction rates. However, temperatures above a certain point will denature the enzymes and slow the reaction rate. Lower temperatures follow the same logic except they lower the kinetic energy of the enzymes and substrates, which slows enzyme-catalyzed reactions (Becker *et al.*, 2009).

The optimal temperature is the point where enzymes are working at their maximum rate; thus, the plant's metabolic activity is working at its maximum rate. This high metabolic level allows for fungal growth to be maximized. Greater hyphal strand growth allows for a greater chance of making contact with the moss. Appressoria formation depends on contact with surfaces (Liu and Kolattukudy, 1999). Within this report, 25°C was the optimum temperature for *P. irregulare* appressoria formation (Fig.

11a). Cantrell and Dowler (1971) support my findings by noting that *P. irregulare* colony diameters were highest between 25 and 30°C.

Moreover, most appressoria were found on *A. serpens* leaves (Fig. 11b). Two reasons can account for this. First, leaves have the greatest surface area on the moss. A large surface area increases the likelihood that hyphal strands will make contact. Thus, it makes sense that the leaves had the most appressoria on them. Second, leaves are one cell layer thick and appressoria can be clearly seen with a light microscope. On the other hand, the stems are many cell layers thick and obstructed the light from the microscope. Thus, the stems were dark colored, which could have hidden some appressoria. But overall, most appressoria were found on moss leaves because of their large surface area and one cell layer thickness.

P. irregulare appressoria increased 48 hours after exposure to A. serpens

As more time passed after exposure of *P. irregulare* to *A. serpens*, more and more appressoria were formed. There was a 67.4% increase of appressoria from 24 hours to 48 hours after exposure (Fig. 12). This finding makes sense when we consider *P. irregulare* growth. When *P. irregulare* was placed next to *A. serpens*, the fungus had to acclimate to the new surroundings and the low nutrient BCD medium. These factors slowed fungal growth, which may have caused less appressoria to be formed. However, more appressoria were formed once *P. irregulare* became acclimated to the new environmental conditions. I hypothesize that less appressoria would be formed once the fungus has exhausted moss cells of their intracellular contents. But, future research would have to be undertaken to support this claim.

Pythium irregulare re-exposure had less appressoria, and most appressoria were on the leaves of A. serpens regardless of P. irregulare exposure

The primary exposure of *P. irregulare* to *A. serpens* had a mean of 7.333 appressoria (Fig. 14). The re-exposure had a smaller mean of 5.857. Although there was no statistical difference, larger sample sizes should be used to better evaluate the influence of exposure on preventing future infections. Like the optimum temperature experiment, most appressoria were found on moss leaves in both exposures (Figs. 15 and 16).

In the primary exposure there was not a statistical difference between appressoria location. However, the re-exposure had a statistical difference between appressoria location. More appressoria were found on leaves than on the stems. In support of my hypothesis, this data suggests that a signal generated at the primary exposure location is traveling through the stem to the site of the re-exposure experiment. At the site of the re-exposure experiment, less appressoria should be formed on the stem because the stem first receives the signal and then the leaves do. This experiment was only 24 hours long, with extended time there might be less appressoria formed on the leaves as well.

Chemical defense mechanisms independent of HR and SAR could be the reason why appressoria formation is lower upon re-exposure. Specifically, the chemical cyclic adenosine 3', 5' monophosphate (cAMP) could be the mediator of this appressoria inhibition. Within the moss, cAMP is a mediator of many cellular processes, including metabolic reactions. Handa and Johri (1977) reported that cAMP was present in the moss *F. hygrometrica*, and seems to regulate specific cellular differentiation processes. Of significance is that Lapp *et al.* (1979) reported cAMP inhibits appressoria formation of the fungus *Colletotrichum graminicola*. This fungus is a plant pathogen responsible for

fungal diseases in many cereal crops. I hypothesize that upon initial infection by *P. irregulare*, certain cellular processes that increase levels of cAMP in and around *A. serpens* were up-regulated. This higher concentration of cAMP inhibits appressoria formation in the re-exposure of *P. irregulare*. In addition, it is important to note that secretion of chemicals by mosses to defend against pathogens does occur. Kato-Noguchi (2009) noted that the chemicals momilactone A and B are secreted by the moss *Hypnum plumaeforme* upon pathogen attack. Interestingly, jasmonic acid (JA), a chemical important for defense responses including SAR, seems to be a mediator of cAMP production. Overall, cAMP is produced by certain mosses and does inhibit appressoria formation.

Future research must be completed in order to support or reject this cAMP hypothesis. First, it must be determined if *A. serpens* produces cAMP. To the best of my knowledge, only a small number of mosses have been tested (Lapp *et al.*, 1979). Then, cAMP has to be applied to *P. irregulare*. Appressoria must be counted before and after cAMP application. If a reduction in appressoria is noted, cAMP levels in *A. serpens* before and after *P. irregulare* exposure have to be determined. If appressoria counts were lower, then I would predict that cAMP levels would be elevated. Overall, a simply structured experiment could be completed to support or reject this hypothesis.

SA does not influence A. serpens or P. irregulare growth

It is not clear whether or not SA influences *A. serpens* growth. Application of 25 and 1000μM SA resulted in *A. serpens* growth that mirrored the moss control (Fig. 19). However, 50, 100, and 500μM SA resulted in reduced *A. serpens* growth.

This inconclusive growth data corresponds with past research. SA is a plant hormone that helps regulate flowering, heat production, growth, and other aspects. However, its influence on growth is not consistent. Some reports note an increased rate of growth with SA treatment. For example, Fariduddin *et al.* (2003) saw an increase in the dry mass in the vascular plant *Brassica juncea* with application of 10^{-5} M SA. However, concentrations above 10^{-5} M lowered dry mass. Also, wheat seedlings had more leaves when applied with 10^{-5} M SA (Hayat *et al.*, 2005). However, SA has been reported to inhibit plant growth. Pancheva *et al.* (1996) noted that SA application to barley seedlings reduced the number of leaves in a concentration dependent manner. Likewise, Christianson and Duffy (2002) noted a decrease in gametophyte bud formation in the moss *F. hygrometrica* with increasing SA concentrations. Clearly, the influence of SA on vascular and non-vascular plant growth is a complex process that is not completely understood.

Future research could reexamine the influence of SA on *A. serpens* growth. The same concentrations of 25, 50, 100, 500, and 1000μM SA should be applied to *A. serpens,* but more moss specimens should be used. Growth measurements should be taken with a digital camera attached to a microscope before and after SA application.

My data suggests the influence of SA on *P. irregulare* growth is more conclusive. Both 500 and 1000μM SA application to *P. irregulare* had similar growth measurements to the controls (Fig. 20). All growth measurements were close to 11.505mm per 7 days.

Interestingly, these findings are not similar to past research. Some research showed that SA inhibits fungal growth, whereas, other research showed enhanced growth. Amborabé *et al.* (2002) noted that SA treatment of 100μM on the fungus *Eutypa*

lata inhibited growth in a concentration dependent manner. Specifically, 1000μM SA treatment inhibited growth completely, and 2000μM SA treatment killed the fungus. However, Kilaru *et al.* (2007) noted that SA concentrations ranging from 0.1-10μM increased hyphal growth in the plant pathogen *Moniliophthora perniciosa*. SA application of 1.0μM increased fungal growth the most. Clearly, past research shows that the influence of SA on fungal growth is contradictory, suggesting that the influence of SA of fungal growth needs to be assessed for each species of fungus.

Future research could help clarify the inconsistencies. However, instead of only applying 25, 50, 100, 500, and 1000μM SA to *P. irregulare*, past research suggests smaller concentrations in the range of 0.1-10μM should also be tested. These SA concentrations would be applied to fungal cultures. The hyphal growing fronts would be measured before and after treatment with SA. This more comprehensive testing could give better insights into the influence of SA on *P. irregulare* growth.

The mean number of appressoria penetrations of A. serpens cells decreased with re-exposure of P. irregulare, and SA concentrations of 25, 100, and 1000μM had an undetermined influence on P. irregulare appressoria penetration events on A. serpens leaf cells

First, the mean number of *P. irregulare* penetrated moss cells in the primary exposure was 2.57 (Fig. 18). The mean number of penetrated cells in the re-exposure was 1.71. This constitutes a 33.5% decrease in *A. serpens* cell penetrations.

Although there was not a significance difference in the number of penetrations between the primary and re-exposure experiments, *A. serpens* SAR could be responsible for this decrease because lots of variability among moss specimens was observed (Fig. 17). Briefly, HR and SAR are intimately linked. HR is the intentional killing of healthy moss cells around the site of pathogen infection. This functions to prevent pathogen

dissemination throughout the moss. The chemicals JA, SA, and ethylene are thought to mediate HR and initiate SAR (Oliver *et al.*, 2009). SAR induces cellular processes within the plant that prevent future pathogen infection. In humans, this is analogous to getting a vaccination to prevent future disease. HR and SAR are distinct but intertwined processes that protect plants from pathogen invasion and possible death.

Second, it was not clear what influence 25, 100, and 1000μM SA treatments had on *P. irregulare* penetrations events (Fig. 21). SA treatments alone seemed to decrease cell penetrations in a concentration dependent manner, but the control negated these findings. Future research into SAR chemical response in *A. serpens* is clearly needed.

The SAR response in plants has been highly researched in economically important agricultural plants, but little research has been done in mosses. Oliver *et al.* (2009) researched the defense mechanisms of the moss *P. patens* when exposed to the pathogenic fungus *P. irregulare*. JA levels within *P. patens* were measured 2, 4, 8, and 24 hours after *P. irregulare* exposure. Researchers observed a 5-fold increase in JA after 24 hours, but no increase in SA levels. Moreover, genetic upregulation of defense related genes were noted upon pathogen exposure. Specifically, the up-regulated genes were *Chs*, *Pal*, and *Lox*. The times of highest expressions for these genes varied between 2 and 24 hours after exposure. Finally, JA, a JA precursor (12-oxo-phytodienoic acid), and a JA derivative (methyl ester methyl jasmonate) were applied to *P. patens*. It was determined that the *Pal* gene was up-regulated by all of these applications. Overall, the Oliver *et al.* (2009) report was the most comprehensive report that dealt with moss SAR.

Key aspects in Oliver *et al.* (2009) and other reports focusing on vascular plants support the results of my experiment. First, it is important to note that *P. irregulare* is a

necrotrophic plant pathogen. Necrotrophic pathogens infect and kill host cells. Thomma *et al.* (2001) and Ton *et al.* (2002) noted that the SA-dependent resistance pathway is activated with biotrophic pathogens. Biotrophic pathogens infect but do not kill host cells. Again, Thomma *et al.* (2001) and Ton *et al.* (2002) showed that the JA and ethylene dependent-resistance pathways are activated with necrotrophic pathogens. Thus, this research supports the inconclusive nature of my SA trials. SA does not have an effect on the infection process in *A. serpens*.

Second, prior research supports why *A. serpens* specimens were never fully killed by *P. irregulare*. I hypothesize that the JA and ethylene dependent resistance pathways were up-regulated in *A. serpens* when exposed to the necrotrophic pathogen *P. irregulare*. Thomma *et al.* (2001) noted that JA and ethylene-dependent pathway activations led to decreased infection of *A. thaliana* by necrotrophic fungi and some bacteria. Clearly, the inconclusive nature of SA testing and lack of complete moss death support my hypothesis that *A. serpens* does have SAR responses.

Future research on SAR

Future research avenues concerning the SAR response in *A. serpens* are numerous and promising. First, application of JA and/or ethylene with *P. irregulare* exposure must be completed. I hypothesize that cell penetrations in *A. serpens* would be lowered significantly. Second, resistance genes up-regulation could be investigated. RNA gel blot analysis of *A. serpens* after SA, JA, and ethylene exposure could be completed. This would provide greater insights into the genetic mechanisms of SAR activation. Finally, SA, JA, and ethylene treated *A. serpens* specimens could be exposed to a myriad of necrotrophic and biotrophic pathogens. This would be important because Roetschi *et al.*

(2001) have noted that these three pathways do not defend against all pathogens. Trends and generalizations about *A. serpens* broad-spectrum pathogen defense could be noted. Overall, the future research possibilities of the SAR response in *A. serpens* are numerous.

A. serpens chemical and protein profile identifications were unsuccessful

If SA induced changes in gene expression, then GC-MS CO_2 extracted compounds and protein profiling should reveal differences in before and after SA treatments. Unfortunately, my attempts were not successful because of limited time. However, future research for both these aspects is promising. First, chemical extraction using supercritical CO_2 is an effective extraction method for plant material. For example, Barton *et al.* (1992) and Sovová *et al.* (1994) used supercritical CO_2 to isolate plant chemicals such as caraway (*Carum carvi*) essential oil and chemicals in peppermint (*Mentha piperita*). Experimental adjustments can be made to optimize chemical isolation and identification in *A. serpens*. This could allow up-regulated SAR chemicals to be determined. Second, *A. serpens* protein identification could provide useful information. Noting protein differences before and after *P. irregulare* exposure or application of JA, SA, or ethylene would provide better insight into the impacts of SAR on proteins as a result in changes of gene expression. Clearly, chemical and protein identifications would be useful to understand the details of SAR in *A. serpens*.

Conclusion

In conclusion, the major findings in this report include that *P. irregulare* does infect *A. serpens* but entire moss death was not noted, 25°C is the optimum temperature for *P. irregulare* appressoria formation, most appressoria were found on *A. serpens* leaves, and the SAR response within *A. serpens* seems to be reducing the number of *P.*

irregulare penetrated moss cells. Future research on the pathogen defense mechanisms of *A. serpens* is important and promising. When we elucidate these complex chemical processes in "simpler" model systems such as *A. serpens*, we can then apply this information to economically important agricultural plants. This will aid farmers and our food system by preventing lower crop yields due to pathogen damage. I look forward to reading about these future experiments and seeing how this research will benefit our society and food system as a whole.

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